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L6	L4 and "pBA-5a"	0	L6
L5	L4 and "TK-1"	0	L5
L4	5681746 [pn]	3	L4
L3	L2 and resist$ near5 complement	6	L3
L2	L1 and factor$ near3 VIII	496	L2
L1	retrovir$ near5 vector$	6772	L1

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RESIST$(RESISTANCE.IADDEND.IADD.89).P29-P90,P92-P94,P24-P28,P21-P23,P1-P19,P20-P20.

Search Results -

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DB=USPT,PGPB,JPAB,EPAB,DWPI,TDBD; PLUR=YES; OP=OR			
L3	L2 and resist$ near5 complement	6	L3
L2	L1 and factor$ near3 VIII	496	L2
L1	retrovir$ near5 vector$	6772	L1

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☑ 1. Document ID: US 6329199 B1

L3: Entry 1 of 6 File: USPT Dec 11, 2001

US-PAT-NO: 6329199
DOCUMENT-IDENTIFIER: US 6329199 B1

TITLE: Retroviral vectors produced by producer cell lines resistant to lysis by human serum

DATE-ISSUED: December 11, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pensiero; Michael	Dickerson	MD	20842	
Collins; Mary K. L.	London W1R 3LN			GBX
Cosset; Francois-Loic	London SW6 3UU			GBX
Takeuchi; Yasuhiro	London SW18 5JJ			GBX
Weiss; Robin A.	London N3 1SS			GBX

US-CL-CURRENT: 435/320.1; 435/325, 435/352, 435/357, 435/363, 435/366, 435/368, 435/369, 435/371, 435/455, 435/456, 536/23.1, 536/23.72, 536/24.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 6183993 B1

L3: Entry 2 of 6 File: USPT Feb 6, 2001

US-PAT-NO: 6183993
DOCUMENT-IDENTIFIER: US 6183993 B1

TITLE: Complement-resistant non-mammalian DNA viruses and uses thereof

DATE-ISSUED: February 6, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Boyce; Frederick M.	Belmont	MA		
Barsoum; James G.	Lexington	MA		

US-CL-CURRENT: 435/69.7; 424/246.1, 435/235.1, 435/456, 435/69.1, 536/23.4, 536/23.71

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC
Draw Desc | Image

☐ 3. Document ID: US 5955441 A

L3: Entry 3 of 6 File: USPT Sep 21, 1999

US-PAT-NO: 5955441
DOCUMENT-IDENTIFIER: US 5955441 A

TITLE: Genetic inhibition of complement mediated inflammatory response

DATE-ISSUED: September 21, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sims; Peter J.	Oklahoma City	OK		
Bothwell; Alfred L.M.	Guilford	CT		

US-CL-CURRENT: 514/44

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC
Draw Desc | Image

☐ 4. Document ID: US 5952225 A

L3: Entry 4 of 6 File: USPT Sep 14, 1999

US-PAT-NO: 5952225
DOCUMENT-IDENTIFIER: US 5952225 A

TITLE: Retroviral vectors produced by producer cell lines resistant to lysis by human serum

DATE-ISSUED: September 14, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pensiero; Michael	Dickerson	MD		
Collins; Mary K. L.	London			GBX
Cosset; Francois-Loic	London			GBX
Takeuchi; Yasuhiro	London			GBX
Weiss; Robin A.	London			GBX

US-CL-CURRENT: 435/352; 435/320.1, 435/325, 435/366, 435/369, 435/371

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC
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☑ 5. Document ID: US 5681746 A

L3: Entry 5 of 6 File: USPT Oct 28, 1997

US-PAT-NO: 5681746
DOCUMENT-IDENTIFIER: US 5681746 A

TITLE: Retroviral delivery of full length factor VIII

DATE-ISSUED: October 28, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bodner; Mordechai	San Diego	CA		
De Polo; Nicholas J.	Solana Beach	CA		
Chang; Stephen	Poway	CA		
Hsu; David Chi-Tang	San Diego	CA		
Respess; James G.	San Diego	CA		

US-CL-CURRENT: 435/350; 435/320.1, 435/366, 435/371, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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6. Document ID: US 5573940 A

L3: Entry 6 of 6 File: USPT Nov 12, 1996

US-PAT-NO: 5573940
DOCUMENT-IDENTIFIER: US 5573940 A

TITLE: Cells expressing high levels of CD59

DATE-ISSUED: November 12, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sims; Peter J.	Mequon	WI		
Bothwell; Alfred L. M.	Guilford	CT		

US-CL-CURRENT: 435/362; 424/93.21, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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Terms	Documents
L2 and resist$ near5 complement	6

Display Format: - | Change Format

Previous Page Next Page

L3: Entry 5 of 6 File: USPT Oct 28, 1997

DOCUMENT-IDENTIFIER: US 5681746 A
TITLE: Retroviral delivery of full length factor VIII

Abstract Paragraph Left (1):
Retroviral vectors for directing expression of full length factor VIII in transduced host cells, plasmids encoding the same, and host cells transformed, transfected, or transduced therewith are disclosed. Also disclosed are retroviral particles comprising such retrovital vectors, as are methods for making such particles in suitable packaging cells. Retroviral particles so produced may be amphotropic, ecotropic, polytropic, or xenotropic; alternatively, they may comprise chimeric or hybrid envelope proteins to alter host range. Also described are retrovital particles comprising retroviral vectors for directing full length factor VIII expression which are complement resistant. Pharmaceutical compositions comprising retrovital particles of the invention are also disclosed, as are methods of treating mammals, particularly humans, afflicted with hemophilia.

Brief Summary Paragraph Right (1):
The present invention relates to retroviral-mediated gene therapy. Specifically, the invention relates to recombinant retroviral vectors capable of delivering nucleic acid constructs encoding full length factor VIII to a patient, pharmaceutical compositions comprising such retroviral vectors, and methods of making and using the same.

Brief Summary Paragraph Right (2):
Numerous methods exist for genetically engineering vertebrate cells. Of particular interest are those methods that may be used to engineer mammalian cells, so as to enable the production of large quantities of various polypeptides (such as erythropoietin and factor VIII), as well as to treat various diseases, for instance serious vital infections, cancers, and genetic diseases. One method for successfully introducing nucleic acid molecules into cells involves the use of viral vectors, with vectors derived from retroviruses being prototypic examples.

Brief Summary Paragraph Right (6):
Retroviral vectors (genetically manipulated forms of naturally occurring retroviruses) have a number of important properties, including: (1)efficient entry of genetic material (the vector genome) into cells; (2) an active, efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; and (4) the potential to target to particular cellular subtypes through control of the vector-target cell binding and the tissue-specific control of gene expression. For example, a foreign gene of interest may be incorporated into the retrovirus in place of the normal retrovital RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of the foreign protein by the host cell.

Brief Summary Paragraph Right (7):
Retroviral vectors and various uses thereof have been described in numerous applications, including Mann, et al. (Cell 33: 153, 1983), Cane and Mulligan (Proc. Nat'l. Acad. Sci. USA 81:6349, 1984), Warner, et al. (1991), AIDS Res. Hum. Retroviruses, vol. 7, p.645, Jolly, et al. (1986), Mol. Cell. Bio., vol. 6, p.1141, U.S. Ser. No. 08/136,739, filed Oct. 12, 1993, WO 93/10814, WO 93/15207, and U.S. Ser. No. 08/155,994, filed Nov. 18, 1993. The ability of retroviral vectors to integrate into the genome of replicating vertebrate cells have made them useful for gene therapy purposes (Miller, et al. Methods in Enzymology 217:581, 1993).

Typically, gene therapy involves adding new or additional genetic material to (1) patient cells in vivo or (2) patient cells that have been removed and which, following transduction, are either reintroduced immediately to the patient or expanded ex vivo prior to reintroduction.

Brief Summary Paragraph Right (8):
Hemophilia is a genetic disease characterized by a severe blood clotting deficiency. As such, it will be amenable to treatment by gene therapy. In hemophilia A, an X-chromosome linked genetic defect disrupts the gene encoding factor VIII, a trace plasma glycoprotein which acts as a cofactor in conjunction with factor IXa in the activation of factor X. In humans, the factor VIII gene codes for 2,351 amino acids. The protein has six domains, designated from amino to carboxy terminus as A1, A2, B, A3, C1, and C2, respectively (Wood, et al., Nature 312:330, 1984: Vehar, et al., Nature 312:337, 1984; and Toole, et al., Nature 312:342, 1984), with a deduced molecular weight of about 280 kilo Daltons (kD). The 980 amino acid B domain is deleted in the activated procoagulant form of the protein. Additionally, in the native protein two polypeptide chains, a and b, flanking the B domain, are bound to a divalent calcium cation.

Brief Summary Paragraph Right (9):
The genetic defect causing hemophilia A affects about one in every 10,000 males. Due to the resultant clotting deficiency, those afflicted with the disease suffer severe bleeding episodes due to small injuries, internal bleeding, and joint hemorrhage, which leads to arthropathy, the major cause of morbidity in hemophilia. Normal levels of factor VIII average between 50 to 200 ng/mL of blood plasma (Mannucci, P. M. in Practical Laboratory Hematology, ed. Koepke, J. A., Churchill Livingstone, N. Y., pp:347-371, 1990); however, patients suffering from mild to moderate hemophilia A typically have plasma levels well below 2-60 ng/mL, while levels below about 2 ng/mL result in severe hemophilia.

Brief Summary Paragraph Right (10):
Previously, therapy for hemophilia A involved repeated administration of human factor VIII purified from blood products pooled in lots from over 1000 donors. However, because of the instability of the factor VIII protein, resulting pharmaceutical products using the natural protein typically were highly impure, with an estimated purity by weight (factor VIII to total protein) of approximately 0.04%. Due to the frequency of administration and inability to remove various human pathogens from such preparations, more than 90% of those suffering from hemophilia A were infected in the 1980s with the human immunodeficiency virus (HIV) as a result of their therapy. Many of these HIV infected patients and other HIV negative hemophiliacs have also been infected by Hepatitis B in the same way. Fortunately, recent advances in genetic engineering have lead to the commercial availability of a recombinant form of the protein free from contamination with human pathogens. However, this form of therapy is expensive and chronic. In addition, most hemophilia A patients in the Unites States do not presently receive factor VIII maintenance therapy, but instead only receive the polypeptide prior to activities or events which might cause bleeding, such as surgery, or to treat spontaneous bleeding. Interestingly, this is despite evidence showing that hemophilic arthropathy can be prevented by administering from an early age prophylactic amounts of factor VIII, typically 24-40 IU per kilogram bodyweight, three times a week. Such therapy kept factor VIII concentrations from falling below 1% of normal (Nillson, et al., J. Internal Med. 232:23, 1992). For these reasons, a genetic therapy affording continuous, long term therapeutically effective expression levels or amounts of factor VIII, i.e., to decrease the severity of or eliminate the clotting disorder associated with hemophilia A, would be of great benefit.

Brief Summary Paragraph Right (11):
However, full length factor VIII is encoded by a gene whose cDNA is about 8,800 base pairs (bp) in length (Zatloukal, et al., Proc. Nat'l. Acad. Sci. USA 91:5148, 1994). As retroviral genomes generally contain fewer than 10,000 nucleotides, packaging efficiency falls dramatically when more than about 10,000 nucleotides are present. In most situations, this is not a problem because retroviral vectors comprising a gene of interest (encoding the desired product) generally do not exceed 10 kb. However, because the factor VIII cDNA is much larger than the typical mammalian cDNA, it was considered unlikely that the full length cDNA could be included in a retroviral vector capable of efficient incorporation into an infectious virion, be transmitted to a target cell, and be expressed therein. As a result, to date successful attempts to incorporate a factor VIII cDNA into a retroviral vector have involved deleted forms of the gene, such as that disclosed by Zatloukal, et al,

supra. Such deletions may result in nuclear transcripts which differ from those derived from a full length factor VIII cDNA. As a result, the foreshortened RNA may be processed and transported differently, as might the resultant protein. Indeed, Toole, et al. (Proc. Nat'l. Acad. Sci. USA, 83:5939, 1986) reported that the B domain deleted protein is more easily processed in transduced cells than the full length protein. Hoeben, et al. (Thrombosis and Haeraostasis, 67(3):341, 1992) reported that when retroviral vectors harboring a factor VIII coding region lacking almost all of the B-domain and a neomycin resistence gene were employed to transduce isolated murine bone marrow cells, in vivo factor VIII expression, at either the mRNA or protein level, could not be detected in progenitor cell-derived cells, despite initial transcription immediately after transduction of the progenitors. However, Southern analysis revealed drug resistant cells contained the vector.

Brief Summary Paragraph Right (12):
It is an object of the present invention to provide recombinant retrovital vectors comprising a full length factor VIII cDNA which may be efficiently packaged into infectious retrovital particles. Such retroviral particles may be used to transduce cells either in vivo or ex vivo. Factor VIII expressed from such transduced cells will be processed and transported in a fashion analogous to the expression product of a normally functioning factor VIII gene. As such, retroviral particles harboring such vectors will be useful in the treatment of hemophilia A.

Brief Summary Paragraph Right (13):
Briefly stated, the present invention provides retroviral vectors directing the expression of a full length factor VIII polypeptide, retroviral particles comprising such vectors, as well as methods of making and using the same. In one aspect of the present invention, retroviral vectors directing the expression of a full length factor VIII polypeptide in transfected host cells are provided. In various embodiments of this aspect of the invention, the retroviral vector is derived from a retrovirus selected from the group consisting of MoMLV, GALV, FeLV, and FIV.

Brief Summary Paragraph Right (14):
Another embodiment concerns retroviral vectors wherein the full length factor VIII polypeptide is encoded by a nucleic acid molecule having a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 1, except that a uracil ("U") replaces every thymidine ("T"), a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence set forth in SEQ ID NO: 1, and nucleotide sequences which, but for the degeneracy of the genetic code, would hybridize to such nucleotide sequences.

Brief Summary Paragraph Right (15):
In another embodiment, such retroviral vectors comprise a promoter selected from the group consisting of a retroviral LTR promoter, a SV40 promoter, a CMV MIE promoter, and a MPMV promoter, wherein the promoter is operably associated with the nucleic acid molecule encoding a full length factor VIII polypeptide. In preferred embodiments, the retroviral vector comprises a retroviral backbone derived from MoMLV encoding a full length factor VIII polypeptide, wherein the full length factor VIII polypeptide is encoded by a nucleic acid molecule having a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 1, except that a uracil ("U") replaces every thymidine ("T"); a nucleotide sequence which hybridizes under stringent conditions to such a nucleotide sequence; and nucleotide sequences which, but for the degeneracy of the genetic code, would hybridize to the foregoing sequences.

Brief Summary Paragraph Right (16):
Another aspect of the invention relates to host cells transfected or transduced by a retroviral vector directing the expression of a full length factor VIII polypeptide. In one embodiment, such host cells are transfected or transduced by a retroviral vector comprising a retroviral backbone derived from MoMLV encoding a full length factor VIII polypeptide, wherein the full length factor VIII polypeptide is encoded by a nucleic acid molecule having a nucleotide sequence selected from the group consisting of a nucleotide sequence set forth in SEQ ID NO: 1, except that a uracil ("U") replaces every thymidine ("T"); a nucleotide sequence which hybridizes under stringent conditions to such a nucleotide sequence; and nucleotide sequences which, but for the degeneracy of the genetic code, would hybridize to any of the the foregoing sequences. In one such embodiment of this aspect of the invention, host cells are packaging cells and further comprise one or more nucleic acid molecules encoding retroviral structural polypeptides. Especially preferred are packaging cells wherein the retroviral structural polypeptides comprise env, pol, and gag

polypeptides.

Brief Summary Paragraph Right (17):
In yet another aspect of the invention, retroviral particles comprising retroviral vectors capable of directing expression of a full length factor VIII polypeptide are also provided herein. Various embodiments of this aspect of the invention provide for retroviral particles that are either amphotropic, ecotropic, polytropic, or xenotropic retroviral particles. In another embodiment, such retroviral particles are resistant to inactivation by a mammalian complement system, particularly a human complement system.

Brief Summary Paragraph Right (18):
Still another aspect of the invention concerns methods of making such retroviral particles comprising transducing and transfecting a packaging cell with a nucleic acid molecule encoding a retroviral vector for directing the expression of full length factor VIII and cultivating a packaging cell under appropriate conditions such that copies of the retroviral vector are produced and incorporated into infectious retroviral particles.

Brief Summary Paragraph Right (19):
In yet another aspect of the invention, pharmaceutical compositions comprising retroviral particles comprising retroviral vectors capable of directing the expression of a full length factor VIII polypeptide in host cells transduced or transfected with the retroviral vectors are provided. In one embodiment, such compositions are lyophilized. In another embodiment, the pharmaceutical compositions comprise retroviral particles according to the invention and a pharmaceutically acceptable diluent. In yet a further aspect of the invention, methods are provided for treating mammals afflicted with hemophilia wherein the mammals are administered a therapeutically effective amount of a retroviral vector produced in accordance with the invention. In the preferred embodiment of this aspect of the invention, the mammal being treated is human and is afflicted with hemophilia A. In another preferred embodiment, a human afflicted with hemophilia A is treated by administering to the patient a therapeutically effective amount of a retroviral particle, preferably in a pharmaceutical composition comprising the retroviral particle in a pharmaceutically acceptable diluent.

Brief Summary Paragraph Right (20):
Another embodiment of this invention concerns retroviral particles comprising a nucleic acid molecule encoding a full length factor VIII polypeptide wherein the full length factor VIII polypeptide comprises an amino acid sequence selected from the group consisting of canine, feline, bovine, monkey, murine, ovine, avian, equine, porcine, rabbit, rat, and human full length factor VIII.

Brief Summary Paragraph Right (21):
In yet another aspect of the invention, plasmids comprising a nucleic acid molecule encoding a retroviral vector for directing the expression of a full length factor VIII polypeptide in host cells transduced or transfected with such plasmids are provided.

Brief Summary Paragraph Right (22):
Yet another aspect of the invention relates to methods for in vivo production of a full length factor VIII polypeptide wherein retroviral vectors capable of directing the expression of a therapeutically effective amount of a full length factor VIII polypeptide are delivered to the cells of a patient. In a preferred embodiment of this aspect, the retroviral vector is delivered to cells by a retroviral particle comprising the retrovital vector. In a preferred embodiment, the retroviral particle targets the delivery of the retroviral vector to specific subsets of cells in the patient. Especially preferred subsets of cells include hematopoietic cells, endothelial cells, liver cells, and combinations thereof. Preferred hematopoietic cells are stem cells from bone marrow or umbilical cord blood. Such methods involve either ex vivo or in vivo delivery of retroviral vectors to the cells. Particularly preferred methods of in vivo delivery of the retroviral vectors according to the invention include parenteral administration and pulmonary administration. In a particularly preferred embodiment of this aspect of the invention, the in vivo production of full length factor VIII results from stable expression of the full length factor VIII polypeptide from a proviral from of the retroviral vector.

Brief Summary Paragraph Right (23):
Another aspect of the invention involves host cells that stably express full length

factor VIII following transduction with a retroviral vector capable of directing the expression of a full length factor VIII polypeptide. In a preferred embodiment, such host cells are of human origin.

Drawing Description Paragraph Right (1):
FIG. 1 is a graphic representation of two retroviral vectors, JW-2 and ND-5, encoding full length factor VIII.

Drawing Description Paragraph Right (3):
FIG. 3 diagrams in vivo factor VIII processing. The full length factor VIII translation product is shown, including the 19 amino acid leader peptide (hatched region) at the N-terminus ("N"). Acidic regions between the A1 and A2 domain and the B and A3 domains are shaded. Cleavage points are indicated by amino acid number. Cleavage by thrombin is indiacted by "IIa". "h.c." and "l.c." represent the heavy and light chains, respectively. Numbering in the various boxes represents relative molecular weights in kD. "PL" means "phospholipid."

Drawing Description Paragraph Right (4):
FIG. 4(A-B). Graph 4A shows factor VIII expression in primary human fibroblasts as measured by Coatest assay. Samples 1 and 2 represent untransduced controls and samples 3-6 are expression levels from fibroblasts transduced with 0.44 mL, 0.133 mL, 0.400 mL, and 1.2 mL of supernatant containing HX/JW-2, respectively. Graph 4B is a Coatest standard curve.

Detailed Description Paragraph Right (2):
"Factor VIII" is a nonenzymatic cofactor found in blood in an inactive precursor form. Precursor factor VIII is converted to the active cofactor, factor VIIIa, through limited proteolysis at specific sites by plasma proteases, notably thrombin and factor IXa. The majority of factor VIII circulates as a two-chain heterodimer most likely due to intracellular or pericellular processing of the single chain gene product. The two chains are noncovalently associated in a metal ion dependent manner.

Detailed Description Paragraph Right (3):
The "biological activity" of factor VIII refers to a function or set of functions performed by the polypeptide or fragments thereof in a biological system or in an in vitro facsimile thereof. In general, biological activities can include effector and cofactor activities. Effector activities include binding of factor VIII or its fragments to other proteins or cells. Effector activity may enhance or be required for cofactor activity. Cofactor activities include enhancement of activation of factor X by factor IXa("tenase"), and possibly the enhancement of inactivation of factors Va or VIIIa by activated protein C. The biological activity of factor VIIIa may be characterized by its ability to form a membrane binding site for factors IXa and X in a conformation suitable for activation of the latter by the former, and possibly by the ability of the B domain of precursor factor VIII to act synergistically with protein S to enhance inactivation of factors Va or VIIIa by activated protein C. This would include standard assays of factor IX or X activation, binding to phospholipids, von Willebrand factor, or specific cell surface molecules, and susceptibility to thrombin, factor IXa, activated protein C, or other specific proteases under appropriate conditions, and correcting the coagulation defect in plasma derived from individuals afflicted with hemophilia A or the prothromboticn defect in individuals allicted with activated proteinase C resistance.

Detailed Description Paragraph Right (4):
A "factor VIII cDNA molecule" is one encoding a full length factor VIII polypeptide. The human full length factor VIII coding region is 7,056 nucleotides.

Detailed Description Paragraph Right (5):
A "full length factor VIII" polypeptide refers to a protein comprising at least 95% of the amino acid sequence, or 2215 amino acids, shown in SEQ ID NO: 1. Also included within this definition are various factor VIII analogues or modified forms comprising at least 95% of the amino acid sequence, or 2215 amino acids, of full length factor VIII, wherein one or more amino acids have been substituted, deleted, or inserted, as is discussed in more detail below. Any such analogue will have at least one of the recognized biological activities of factor VIII. Nucleic acids encoding full length factor VIII refer to those encoding a full length factor VIII polypeptide.

Detailed Description Paragraph Right (11):
A "unique nucleic acid fragment" is one comprising a contiguous nucleotide sequence that is not known to exist in another nucleic acid molecule. Unique fragments can be identified by selecting particular nucleotide sequences found in a factor VIII coding region and comparing such sequences to those found in various nucleotide sequence databases, including Genbank (available from the National Center for Biotechnology Information [NCBI], European Molecular Biology Library [EMBL]), and GeneSeq.TM. (Intelligenetics, Inc., Mountain View, Calif.) using publicly available computer algorithms such as FASTA.TM. (Genetics Computer Group, Madison, Wis.) and BLAST (NCBI).

Detailed Description Paragraph Right (12):
"Vector construct", "retroviral vector", "recombinant vector", and "recombinant retroviral vector" refer to a nucleic acid construct capable of directing the expression of a full length factor VIII gene. The retroviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. In addition, the retrovital vector must include a nucleic acid molecule which, when transcribed in the presence of a full length factor VIII gene, is operably linked thereto and acts as a translation initiation sequence. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the vector construct may also include a signal which directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way example, such vectors will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. In order to express a full length factor VIII polypeptide from such a vector, a full length factor VIII ceding region is also included.

Detailed Description Paragraph Right (14):
The present invention is based on the unexpected discovery that retroviral vectors comprising a nucleic acid molecule encoding full length factor VIII can be efficiently packaged into infectious retroviral particles and that cells transduced in vivo with such vectors produce biologically active factor VIII. As a result, retroviral vectors encoding full length factor VIII can be used for purposes of gene therapy. A more thorough description of such retroviral vectors, their production and packaging, and uses therefore is provided below.

Detailed Description Paragraph Right (15):
In humans and other mammals examined, the factor VIII gene is known to be located on the X chromosome and span more than about 186 kb (kilobases). Transcription of the gene results in the eventual production of a mRNA of approximately 8,800 nucleotides encoding the full length polypeptide. The nucleotide sequence of the factor VIII coding region is presented in SEQ ID NO: 1 and has been published in various locations. For instance, see Wood, et al. (Nature, 312:330, 1984; U.S. Pat. No. 4,965,199). The coding region spans 7,056 nucleotides, exclusive of 5' and 3' untranslated sequences, but for the translation termination codon TGA. Allelic variants of this sequence encoding biologically active, full length factor VIII likely exist and may also be used in the practice of this invention. Such allergic variants may contain differences only detectable at the nucleic acid level, i.e., due to conservative nucleotide substitutions. On the other hand, they may be manifest by one or more amino acid differences in the overall sequence, i.e., by deletions, insertions, substitutions, or inversions of one or more amino acids. However, no such variant will comprise less than about 95% (by number) of the nucleotides of SEQ ID NO: 1.

Detailed Description Paragraph Right (16):
In vivo, a major site of factor VIII production is thought to be the liver, but factor VIII mRNA has also been detected in the spleen, kidney and lymph nodes [White, et al., Blood, 73: 1, 1989]. However, other cell types which do not normally express the protein can express the polypeptide, including smooth muscle cells of the primary vasculature [Powell, et al., FEBS Letters, 303(2,3):173, 1992]. As a result, hematopoietic cells are particularly attractive gene therapy targets [Hoeben, et al., supra].

Detailed Description Paragraph Right (17):

Native human full length factor VIII is a heat labile single chain glycoprotein comprising 2351 amino acids, with the N-terminal 19 residues functioning as a leader peptide that is later cleaved. The remaining 2332 residues comprise six distinct domains, arranged as follows: A1-A2-B-A3-C1-C2. The A domains (each about 330 amino acids in length) share homology with factor V and the plasma copper binding protein ceruloplasmin. Similarly, the two C domains (each about 150 amino acids) are homologous to those of factor V and other phospholipid binding proteins. The B domain contains 19 of the 25 potential sites (Asn-X-Ser/Thr) for N-linked glycosylation, although it is not required for procoagulant activity. During intracellular processing prior to secretion, the polypeptide is cleaved after residues 1313 and 1648 to generate heavy ("a") and light ("b") chains, respectively. The observed relative molecular weight of the a chain is about 200 kD, as measured by SDS-PAGE, and that of the b chain is about 80 kD. The two chains then assemble in a non-covalent complex around a divalent metal ion.

Detailed Description Paragraph Right (18):
During processing, factor VIII is also sulfated on six Tyr residues (amino acid residues 346, 718, 719, 723, 1664, and 1680). Sulfation is required for full functional activity, but not for synthesis or secretion (Pittman, et al., Biochemistry, 31:3315, 1992). Huttner, et al., Mol. Cell. Biol., 6:97, 1988) proposed a consensus sequence for tyrosine sulfation, corresponding to 7 potential sulfation sites in full length factor VIII. Many proteins known to interact with thrombin, such as hirudin, fibrinogen, heparin cofactor II, bovine factor X, vitronectin, factor V, and factor VIII, have one or more sulfated tyrosine residues. In hirudin, Tyr sulfation in the C-terminal region increases binding affinity to the anion binding exosite of thrombin (Rydel, et al., Science, 249:277, 1990; Niehrs, et al., J. Biol. Chem., 262:16467, 1990). All sites which are sulfated in factor VIII border thrombin, factor IXa, or activated protein C cleavage sites. Using various techniques, for instance, site directed mutagenesis, nucleic acids encoding full length factor VIII having fewer or additional sulfation sites can be readily generated.

Detailed Description Paragraph Right (19):
Prior to activation, factor VIII circulates in plasma bound to yon Willebrand factor (vWf), which stabilizes it. Factor VIII has a plasma half-life of about 12 hr. Factor VIII and vWf circulate in plasma as a non-covalently linked complex. vWf is necessary for mediating platelet-vessel interactions at sites of vascular injury (Saenko, et al., J. Bio. Chem., 269(15):11601, 1994). The factor VIII heavy chain is minimally represented by the A1-A2 domains, and it exhibits heterogeneity due the presence of some or all of the contiguous B domain. The light chain corresponds to the A3-C 1-C2 domains and contains sites for binding vWf (Lollar, et al., J. Biol. Chem., 263:10451, 1988; Hamer, et al., Eur. J. Biochem., 166:37, 1987), activated protein C (Walker, et al., J. Bio. Chem., 265:1484, 1990), and phospholipids (Foster, et al., Blood, 75:1999, 1990; Bloom, J. W. Thromb. Res., 48:439, 1987). vWF prevents factor VIII from binding to phospholipids and platelets (Fay, et al., J. Biol. Chem., 266:2172, 1991; Nesheim, et al., J. Biol. Chem., 266:17815, 1991). Upon activation by thrombin, factor VIIIa dissociates from vWf (Lollar, et al., supra). A polypeptide comprising only the C2 domain, and expressed in E. coli binds to phosphatidylserine or vWf in a dose dependent manner. The vWf binding sire was localized to amino acids 2303 to 2332, and its occupancy is also known to prevent factor VIII-phosphatidylserine binding (Foster, et al., supra). Residues 1673-1689 (part of the light chain acidic region) and sulfated Tyr.sup.1680 may also be required for high affinity binding of vWf to the factor VIII light chain (Leyte, et al., J. Biol. Chem., 266:740, 1991), as thrombin cleavage at residue 1689 leads to loss of vWf binding.

Detailed Description Paragraph Right (20):
Factor VIII has two thrombin cleavage sites, between Arg.sup.739 and Ser.sup.740 and between Arg.sup.1689 and Ser.sup.1690 (Toole, et al., supra), yielding a 90 kD heavy chain and 73 kD light chain. Factor VIIIa acts as a cofactor with factor IXa(activated by factor XIa or VIIa), calcium ions, and phospholipids to activate factor X to form factor Xa, potentially on the surface of platelets or endothelial cells. Thrombin cleavage activates the procoagulant activity of factor VIII 20- to 200-fold. Factor VIIIa is then inactivated by various proteolytic activities. See FIG. 3 for a depiction of factor VIII processing.

Detailed Description Paragraph Right (21):
In addition to encoding a full length factor VIII polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2, the present invention also envisions

recombinant retroviral vectors which encode analogues of full length factor VIII wherein one or more amino acids are substituted, deleted, or inserted. Such alterations may provide for improved expression, enhanced stability, presentation of altered functional properties, altered serum half-life and clearance times, different patterns of glycosylation, etc. Representative examples include addition, deletion, or movement of one or more sulfation sites, glycosylation sites, etc. Also, changes may be engineered to improve metal ion binding or thrombin interactions, to introduce novel disulfide bridges to improve stability, etc. In preferred embodiments of the invention, full length factor VIII analogues will retain those sequences required for activation by thrombin. Thrombin activation of various full length factor VIII analogs can be assayed by comparing the kinetics of thrombin activation of native, plasma derived factor VIII versus that of an analog. Activation can be measured using a standard coagulation assay (see Example 3, infra) or a plasma-free tenase assay using purified proteins, among other assays.

Detailed Description Paragraph Right (22):
Nucleic acids encoding full length factor VIII polypeptide analogues will differ in more one more nucleotides as compared to the nucleotide sequence set out in SEQ ID NO: 1. Alterations may be introduced by a variety of techniques, including random mutagenesis, site directed mutagenesis, or solid state nucleic acid synthesis. For example, all or part of the full length factor VIII gene present in a retroviral vector may be modified to contain one or more degenerate codons, i.e., a different codon coding for the same amino acid, preferred for expression in the particular species to be treated. A "codon preferred for expression" in a particular species is a codon which is represented in highly expressed structural genes of that species in a proportion greater than would be randomly expected. In any event, the "preferred" codon will code for the same amino acid as the codon that was replaced due to the degenerate nature of the genetic code. Codon preferences are known for many species, and can be deduced by statistical analysis of codon usage in genes encoding highly expressed proteins in species for which such preferences have not yet been determined. One or more preferred codons can be incorporated into a nucleic acid molecule by various methods, including site directed mutagenesis and partial or complete synthetic gene synthesis. Alternatively, all or part of the gene may be modified to minimize the formation of secondary structures which might reduce the efficiency of translation or post transcriptional processing. For instance, Lynch, et al. (Human Gene Therapy, 4:259, 1993) studied the use of retroviral vectors for transfer and expression of truncated forms of factor VIII lacking part or all of non-essential B-domain sequences. Expression and vital titer were about 100-fold lower than titer and protein production from identical retroviral backbones containing other cDNAs. This reduction correlated with a 100-fold lower accumulation of factor VIII retroviral vector RNAs as compared to other vector RNAs. Analysis revealed the presence of sequences in the factor VIII coding region that may inhibit vector RNA accumulation. One or more of such sequences can be modified using well known techniques.

Detailed Description Paragraph Right (23):
As noted above, the present invention provides compositions and methods comprising recombinant retroviral vectors. The construction of recombinant retroviral vectors is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S. Ser. No. 07/586,603, filed Sep. 21, 1990, which is hereby incorporated by reference in its entirety). These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see U.S. Ser. No. 07/800,921, which is hereby incorporated by reference in its entirety).

Detailed Description Paragraph Right (24):
In the broadest terms, the retroviral vectors of the invention comprise a transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. In addition, the retroviral vector must include a nucleic acid molecule which, when transcribed in the presence of a full length factor VIII gene, is operably linked thereto and acts as a translation initiation sequence. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retrovital vector). Optionally, the vector construct may also include a signal which directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way example, such vectors will typically include a 5' LTR,

a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. Such vectors do not contain one or more of a complete gag, pol, or env gene, thereby rendering them replication incompetent. In addition, nucleic acid molecules coding for a selectable marker are neither required nor preferred.

Detailed Description Paragraph Right (25):
Preferred retroviral vectors contain a portion of the gag coding sequence, preferably that portion which comprises a splice donor and splice acceptor site, the splice acceptor site being positioned such that it is located adjacent to and upstream from the full length factor VIII coding region. In a particularly preferred embodiment, the gag transcriptional promoter is positioned such that an RNA transcript initiated therefrom contains the 5' gag UTR and the full length factor VIII coding region. As an alternative to the gag promoter to control expression of the full length factor VIII coding region, other suitable promoters, some of which are described below, may be employed. In addition, alternate enhancers may be employed in order to increase the level of full length factor VIII expression.

Detailed Description Paragraph Right (26):
In preferred embodiments of the invention, retroviral vectors are employed, particularly those based on Moloney murine leukemia virus (MoMLV). MoMLV is a murine retrovirus which has poor infectivity outside of mouse cells. The related amphotropic N2 retrovirus will infect cells from human, mouse and other organisms. Other preferred retroviruses which may be used is the practice of the present invention include Gibbon Ape Leukemia Virus (GALV) (Todaro, et al., Virology, 67:335, 1975; Wilson, et al., J. Vir., 63:2374, 1989), Feline Immunodeficiency Virus (FIV) (Talbatt, et al., Proc. Nat'l. Acad. Sci. USA, 86:5743, 1984), and Feline Leukemia Virus (FeLV) (Leprevette, et al., J. Vir., 50:884, 1984; Elder, et al., J. Vir., 46:871, 1983; Steward, et al., J. Vir., 58:825, 1986; Riedel, et al., J. Vir., 60:242, 1986), although retroviral vectors according to the invention derived from other type C retroviruses (Weiss, RNA Tumor Viruses, vols. I and II, Cold Spring Harbor Laboratory Press, N.Y.) can also be generated.

Detailed Description Paragraph Right (28):
In another preferred embodiment, the retroviral vector contains a splice donor (SD) site and a splice acceptor (SA) site, wherein the SA is located upstream of the site where the full length factor VIII coding region ("gene") is inserted into the recombinant retroviral vector. In a preferred embodiment, the SD and SA sites will be separated by a short, i.e., less than 400 nucleotide, intron sequence. Such sequences may serve to stabilize RNA transcripts. Such stabilizing sequences typically comprise a SD-intron-SA configuration located 5' to the coding region of full length factor VIII.

Detailed Description Paragraph Right (29):
The recombinant retrovital vectors of the invention will also preferably contain transcriptional promoters derived from the gag region operably positioned such that a resultant transcript comprising the full length factor VIII coding region further comprises a 5' gag UTR (untranslated region) upstream of the factor VIII coding region.

Detailed Description Paragraph Right (30):
In one embodiment, recombinant retroviral vectors comprising a full length factor VIII gene are under the transcriptional control of an event-specific promoter, such that upon activation of the event-specific promoter the full length factor VIII coding region is expressed. Numerous event-specific promoters may be utilized within the context of the present invention, including for example, promoters which are activated by cellular proliferation (or are otherwise cell-cycle dependent) such as the thymidine kinase or thymidylate synthase promoters (Merrill, Proc. Natl. Acad. Sci. USA, 86:4987, 1989; Deng, et al., Mol. Cell. Biol., 9:4079, 1989); or the transferrin receptor promoter, which will be transcriptionally active primarily in rapidly proliferating cells (such as hematopoietic cells) which contain factors capable of activating transcription from these promoters preferentially to express and secrete factor VIII into the blood stream; promoters such as the .alpha. or .beta. interferon promoters which are activated when a cell is infected by a virus (Fan and Maniatis, EMBO J., 8:101, 1989; Goodbourn, et al., Cell, 45:601, 1986); and promoters which are activated by the presence of hormones, e.g., estrogen response promoters. See Toohey et al., Mol. Cell. Biol., 6:4526, 1986.

Detailed Description Paragraph Right (31):

In another embodiment, recombinant retroviral vectors are provided which comprise a full length factor VIII ceding region under the transcriptional control of a tissue-specific promoter, such that upon activation of the tissue-specific promoter the factor VIII gene is expressed. A wide variety of tissue-specific promoters may be utilized within the context of the present invention. Representative examples of such promoters include: liver-specific promoters, such as Phospho-Enol-Pyruvate Carboxy-Kinase ("PEPCK") (Hatzoglou, et al., J. Biol. Chem., 263:17798, 1988; Benvenisty, et al., Proc. Natl. Acad. Sci. USA, 86:1118, 1989; Vaulont, et al., Mol. Cell. Biol., 6:4409, 1989), the alcohol dehydrogenase promoter (Felder, Proc. Natl. Acad. Sci. USA, 86:5903, 1989), and the albumin promoter and the alphafetoprotein promoter (Feuerman, et al., Mol. Cell. Biol., 9:4204, 1989; Camper and Tilghman, Genes Develop., 3:537, 1989); B cell specific promoters such as the IgG promoter; pancreatic acinar cell specific promoters such as the elastase promoter (Swift, et al., Genes Develop., 3:687, 1989) and promoters which are specific for .beta. cells of the pancreas, such as the insulin promoter (Ohlsson, et al., Proc. Natl. Acad. Sci. USA, 85:4228, 1988; Karlsson, et al., Mol. Cell. Biol., 9:823, 1989); breast epithelial specific promoters such as the casein promoter (Doppler, et al., Proc. Natl. Acad. Sci. USA, 86:104, 1989) and the whey (wap) promoter; promoters which regulate skeletal muscle such as the myo-D binding site (Burden, Nature, 341:716, 1989; Weintraub, et al., Proc. Natl. Acad. Sci. USA, 86:5434, 1989); promoters which are specific for the pituitary gland, such as the growth hormone factor promoter (Ingraham, et al., Cell, 55:519, 1988; Bodner, et al., Cell, 55:505, 1988); promoters which are specific for melanosomes, such as the tyrosine hydroxylase promoter; T-cell specific promoters such as the T-cell receptor promoter (Anderson, et al., Proc. Natl. Acad. Sci. USA, 85:3551, 1988; Winoto and Baltimore, EMBO J., 8:29, 1989); bone-specific promoters such as the osteocalcin promoter (Markose, et al., Proc. Natl. Acad. Sci. USA, 87:1701, 1990; McDonnell, et al., Mol. Cell. Biol, 9:3517, 1989; Kerner, et al., Proc. Natl. Acad. Sci. USA, 86:4455, 1989), the IL-2 promoter, IL-2 receptor promoter, and the MHC Class II promoter, and hematopoietic tissue specific promoters, for instance erythoid specific-transcription promoters which are active in erythroid cells, such as the porphobilinogen deaminase promoter (Mignotte, et al., Proc. Natl. Acad. Sci. USA, 86:6458, 1990), .alpha. or .beta. globin specific promoters (van Assendelft. et al., Cell, 56:969, 1989, Forrester, et al., Proc. Natl. Acad. Sci. USA, 86:5439, 1989), endothelial cell specific promoters such as the vWf promoter, magakaryocyte specific promoters such as .beta.-thromboglobulin, and many other tissue-specific promoters.

Detailed Description Paragraph Right (32):
Retroviral vectors according to the invention may also contain a non-LTR enhancer or promoter, e.g., a CMV or SV40 enhancer operably associated with other elements employed to regulate expression of the factor VIII gene. Additionally, retrovital vectors from which the 3' LTR enhancer has been deleted, thereby inactivating the 5' LTR upon integration into a host cell genome, are also contemplated by the invention. A variety of other elements which control gene expression may also be utilized within the context of the present invention, including, for example, locus-defining elements such as those from the .beta.-globin gene and CD2, a T cell marker. In addition, elements which control expression at the level of splicing, nuclear export, and/or translation may also be included in the retrovital vectors. Representative examples include the .beta.-Globin intron sequences, the rev and are elements from HIV-1, the constitutive transport element (CTE) from Mason-Pfizer monkey virus (MPMV), a 219 nucleotide sequence that allows rev-independent replication of rev-negative HIV proviral clones, and a Kozak sequence. Rev protein functions to allow nuclear export of unspliced and singly spliced HIV RNA molecules. The MPMV element allows nuclear export of intron- containing mRNA. The CTE element maps to MPMV nucleotides 8022-8240 a (Bray, et al., Biochemistry, 91:1256, 1994).

Detailed Description Paragraph Right (33):
In a preferred embodiment, retrovital vectors of the invention will include a "cis" element 5' located between the promoter and the full length factor VIII coding region. Such "cis" elements will generally comprise a splice donor and splice acceptor site separated by a short intervening, non-coding sequence. A particularly preferred cis element comprises a splice donor site from CMV and a splice acceptor from immunoglobulin, separated by a short CMV intron sequence, as described below in Example 1.

Detailed Description Paragraph Right (34):
Retroviral vectors according to the invention will often be encoded on a plasmid, a nucleic acid molecule capable of propogation, segregation, and extrachromosomal maintenance upon introduction into a host cell. As those in the art will understand,

any of a wide range of existing or new plasmids can be used in the practice of the invention. Such plasmids contain an origin of replication and typically are modified to contain a one or more multiple cloning sites to facilitate recombinant use. Preferably, plasmids used in accordance with the present invention will be capable of propogation in both eukaryotic and prokaryoric host cells.

Detailed Description Paragraph Right (35):
Another aspect of the invention relates to methods of producing retroviral particles incorporating the retroviral vectors described herein. In one embodiment, vectors are packaged into infectious virions through the use of a packaging cell. Briefly, a packaging cell is a cell comprising, in addition to its natural genetic complement, additional nucleic acids coding for those retroviral structural polypeptides required to package a retroviral genome, be it recombinant (i.e., a retroviral vector) or otherwise. The retroviral particles are made in packaging cells by combining the retroviral genome with a capsid and envelope to make a transduction competent, preferably replication defective, virion. Briefly, these and other packaging cells will contain one, and preferably two or more nucleic acid molecules coding for the various polypeptides, e.g., gag, pol, and env, required to package a retroviral vector into an infectious virion. Upon introduction of a nucleic acid molecule coding for the retroviral vector, the packaging cells will produce infectious retroviral particles. Packaging cell lines transfected with a retroviral vector according to the invention which produce infectious virions are referred to as "producer" cell lines.

Detailed Description Paragraph Right (36):
A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including without limitation, epithelial cells, fibroblasts, hepatocytes, endothelial cells, myoblasts, astrocytes, lymphocytes, etc. Preferentially, cell lines are selected that lack genomic sequences which are homologous to the retroviral vector construct, gag/pol expression cassette and env expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (Martin et al., Proc. Natl. Acad. Sci., USA, vol. 78:4892-96, 1981; and U.S. Ser. No. 07/800,921).

Detailed Description Paragraph Right (37):
The most common packaging cell lines (PCLs) used for MoMLV vector systems (psi2, PA12, PA317) are derived from murine cell lines. However, murine cell lines are typically not the preferred choice to produce retrovital vectors intended for human therapeutic use because such cell lines are known to: contain endogenous retroviruses, some of which are closely related in sequence and retroviral type to the MLV vector system used here; contain non-retroviral or defective retroviral sequences that are known to package efficiently; and cause deleterious effects due to the presence of murine cell membrane components.

Detailed Description Paragraph Right (38):
An important consideration in developing packaging cell lines useful in the invention is the production therefrom of replication incompetent virions, or avoidance of generating replication-competent retrovirus (RCR) [Munchau et al., Virology, vol. 176:262-65, 1990]. This will ensure that infectious retroviral particles harboring the recombinant retrovital vectors of the invention will be incapable of independent replication in target cells, be they in vitro or in vivo. Independent replication, should it occur, may lead to the production of wild-type virus, which in turn could lead to multiple integrations into the chromosome(s) of a patient's cells, thereby increasing the possibility of insertional mutagenesis and its associated problems. RCR production can occur in at least two ways: (1) through homologous recombination between the therapeutic proviral DNA and the DNA encoding the retroviral structural genes ("gag/pol" and "env") present in the packaging cell line; and (2) generation of replication-competent virus by homologous recombination of the proviral DNA with the very large number of defective endogenous proviruses found in the packaging cell line.

Detailed Description Paragraph Right (39):
To circumvent inherent safety problems associated with the use of murine based recombinant retroviruses, as are preferred in the practice of this invention, packaging cell lines may be derived from various non-murine cell lines. These include cell lines from various mammals, including humans, dogs, monkeys, mink, hamsters, and rats. As those in the art will appreciate, a multitude of packaging cell lines can be generated using techniques known in the art (for instance, see U.S. Ser. No. 08/156,789 and U.S. Ser. No. 08/136,739). In preferred embodiments,

cell lines are derived from canine or human cell lines, which are known to lack genomic sequences homologous to that of MoMLV by hybridization analysis (Martin et al., supra). A particularly preferred parent dog cell line is D17 (A.T.C.C. accession no. CRL 8543). HT-1080 (A.T.C.C. accession no. CCL 121; Graham et al., Vir., vol. 52:456, 1973) and 293 cells (Felgner et al., Proc. Nat'l. Acad. Sci. USA 84:7413, 1987) represent particularly preferred parental human cell lines. Construction of packaging cell lines from these cell lines for use in conjunction with a MoMLV based recombiant retroviral vector is described in detail in U.S. Ser. No. 08/156,789, supra.

Detailed Description Paragraph Right (40):
Thus, a desirable prerequisite for the use of retroviruses in gene therapy is the availability of retroviral packaging cell lines incapable of producing replication competent, or "wild-type," virus. As packaging cell lines contain one or more nucleic acid molecules coding for the structural proteins required to assemble the retroviral vector into infectious retroviral particles, recombination events between these various constructs might produce replication competent virus, i.e., infectious retroviral particles containing a genome encoding all of the structural genes and regulatory elements, including a packaging signal, required for independent replication. In the past several years, many different constructions have been developed in an attempt to obviate this concern. Such constructions include: deletions in the 3' LTR and portions of the 5' LTR (see, Miller and Buttimore, Mol. Cell. Biol., vol. 6:2895-2902, 1986), where two recombination events are necessary to form RCR; use of complementary portions of helper virus, divided among two separate plasmids, one containing gag and pol, and the other containing env (see, Markowitz et al., J. Virol., vol. 62:1120-1124; and Markowitz et al., Virology, vol 167: 600-606, 1988), where three recombination events are required to generate RCR.

Detailed Description Paragraph Right (41):
More recently, further improved methods and compositions for inhibiting the production of replication incompetent retrovirus have been developed. See co-owned U.S. Ser. No. 09/028,126, filed Sep. 7, 1994. Briefly, the spread of replication competent retrovirus generated through recombination events between the recombinant retroviral vector and one or more of the nucleic acid constructs coding for the retroviral structural proteins may be prevented by providing vectors which encode a non-biologically active inhibitory molecule, but which produce a nucleic acid molecule encoding a biologically active inhibitory molecule in the event of such recombination. The expression of the inhibitory molecule prevents production of RCR either by killing the producer cell(s) in which that event occurred or by suppressing production of the retroviral vectors therein. A variety of inhibitory molecules may be used, including ribozymes, which cleave the RNA transcript of the replication competent virus, or a toxin such as ricin A, tetanus, or diphtheria toxin, herpes thymidine kinase, etc. As those in the art will appreciate, the teachings therein may be readily adapted to the present invention.

Detailed Description Paragraph Right (42):
In addition to issues of safety, the choice of host cell line for the packaging cell line is of importance because many of the biological properties (such as titer) and physical properties (such as stability) of retroviral particles are dictated by the properties of the host cell. For instance, the host cell must efficiently express (transcribe) the vector RNA genome, prime the vector for first strand synthesis with a cellular tRNA, tolerate and covalently modify the MLV structural proteins (proteolysis, glycosylation, myristylation, and phosphorylation), and enable virion budding from the cell membrane. For example, it has been found that vector made from the mouse packaging line PA317 is retained by a 0.3 micron filter, while that made from a CA line described herein will pass through. Furthermore, sera from primates, including humans, but not that from a wide variety of lower mammals or birds, is known to inactivate retroviruses by an antibody independent complement lysis method. Such activity is non-selective for a variety of distantly related retroviruses. Retroviruses of avian, murine (including MoMLV), feline, and simian origin are inactivated and lysed by normal human serum. See Welsh et al., (1975) Nature, vol. 257:612-614; Welsh et al., (1976) Virology, vol. 74:432-440; Banapour et al., (1986) Virology, vol. 152:268-271; and Cooper et al., (1986) Immunology of the Complement System, Pub. American Press, Inc., pp:139-162. In addition, replication competent murine amphotropic retroviruses injected intravenously into primates in vivo are cleared within 15 minutes by a process mediated in whole or in part by primate complement (Cornetta et al. (1990), Human Gene Therapy, vol. 1:15-30; Cornetta et al. (1991), Human Gene Therapy, vol. 2:5-14). However, it has recently been discovered that retroviral resistance to complement inactivation by human serum is

mediated, at least in some instances, by the packaging cell line from which the retroviral particles were produced. Retroviruses produced from various human packaging cell lines were resistant to inactivation by a component of human serum, presumably complement, but were sensitive to serum from baboons and macques. See commonly owned U.S. Ser. No. 08/367,071, filed on a date even herewith. Thus, in a preferred embodiment of the invention, recombinant retr VIII are partials coding for full length factor VIII are produced in human packaging cell lines, with packaging cell lines derived from HT1080 or 293 cells being particularly preferred.

Detailed Description Paragraph Right (43):
In addition to generating infectious, replication defective recombinant retroviruses as described above, at least two other alternative systems can be used to produce recombinant retroviruses carrying the vector construct. One such system (Webb, et al., BBRC, 190:536, 1993) employs the insect virus, baculovirus, while the other takes advantage of the mammalian viruses vaccinia and adenovirus (Pavirani, et al., BBRC, 145:234, 1987). Each of these systems can make large amounts of any given protein for which the gene has been cloned. For example, see Smith, et al. (Mol. Cell. Biol., 3:12, 1983); Piccini, et al. (Meth. Enzymology, 153:545, 1987); and Mansour et al. (Proc. Natl. Acad. Sci. USA, 82:1359, 1985). These retroviral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes and, hence, could be adapted to make retroviral vector particles from tissue culture. In an adenovirus system, genes can be inserted into vectors and used to express proteins in mammalian cells either by in vitro construction (Ballay, et al., 4:3861, 1985) or by recombination in cells (Thummel, et al, J. Mol. Appl. Genetics, 1:435, 1982).

Detailed Description Paragraph Right (45):
Another important factor to consider in the selection of a packaging cell line is the viral liter produced therefrom following introduction of a nucleic acid molecule from which the retroviral vector is produced. Many factors can limit viral titer. One of the most significant limiting factors is the expression level of the packaging proteins gag, pol, and env. In the case of retroviral particles, expression of retroviral vector RNA from the provirus can also significantly limit titer. In order to select packaging cells and the resultant producer cells expressing high levels of the required products, an appropriate titering assay is required. As described in greater detail below, a suitable PCR-based titering assay has been developed.

Detailed Description Paragraph Right (46):
In addition to preparing packaging and producer cell lines which supply proteins for packaging that are homologous for the backbone of the viral vector, e.g., retroviral gag, pol, and env proteins for packaging of a retroviral vector, packaging and producer systems which result in chimeric viral particles, for instance a MoMLV-based retroviral vector packaged in a DNA virus capsid, may also be employed. Many other packaging and producer systems based on viruses unrelated to that of the viral vector can also be utilized, as those in the art will appreciate.

Detailed Description Paragraph Right (47):
Another aspect of the invention concerns retroviral vectors having an altered host range. The host cell range specificity of a retrovirus is determined in part by the env gene products present in the lipid envelope. Interestingly, envelope proteins from one retrovirus can often substitute, to varying degrees, for that of another retrovirus, thereby altering host range of the resultant vector. Thus, packaging cell lines (PCLs) may be generated to express either amphotropic, ecotropic, xenotropic, or polytropic envelopes. Additionally, retroviruses according to the invention which contain "hybrid" or "chimeric" envelope proteins can be similarly generated. Vector produced from any of these packaging cell lines can be used to infect any cell which contains the corresponding distinct receptor (Rein and Schultz, Virology, 136:144, 1984).

Detailed Description Paragraph Right (49):
Miller et al. (Mol. Cell. Biol., 5:431, 1985) constructed a MoMLV-derived retroviral vector to introduce dihydrofolate reductase into susceptible cells and included the envelope region from the related amphotropic retrovirus 4070A to broaden the host range of the vector. Similarly, envelope proteins from amphotropic, ecotropic, polytropic, and xenotropic retroviruses can be utilized. In addition, alterations in the host range can be effected by including heterologous membrane-associated proteins, i.e., membrane-associated proteins having at least one origin other than a virus of the same viral family as the origin of the nucleocapsid protein of the

vector particle, within a retrovital particle. For instance, vesicular stomatitis virus (VSV), a member of the rhabdovims family, is known to participate in pseudotype formation with retroviruses. See U.S. Ser. No. 07/658,632, filed 19 Feb., 1991.

Detailed Description Paragraph Right (50):
Briefly, in this aspect the present invention provides for enveloped retroviral particles, comprising: a nucleocapsid including nucleocapsid protein having an origin from a first virus, which is a retrovirus; a packageable nucleic acid molecule encoding full length factor VIII associated with the nucleocapsid; and a membrane-associated protein which determines a host range, the membrane-associated protein being from other than a retrovirus of the same taxonomic family as the first retrovirus. Preferably, the membrane-associated protein is from a second virus having a different host range than the first virus, such as a naturally occurring membrane-associated protein, e.g., VSV G protein.

Detailed Description Paragraph Right (55):
In addition to, or in lieu of, tissue targeting, tissue specific promoters can be employed to drive the expression of full length factor VIII in only specific cell types.

Detailed Description Paragraph Right (56):
In order to control the specific site of integration into a patient's genome in those instances where the viral vector employed leads to integration of the viral genome into a chromosome of the recipient cell, as occurs in the case of retroviral infection, homologous recombination or use of a modified integrase enzyme which directs insertion to a specific site can be utilized. Such site-specific insertion of the full length factor VIII gene may provide for gene replacement therapy, reduced chances of insertional mutagenesis, minimize interference from other sequences present in the patient's DNA, and allow insertion at specific target sites to reduce or eliminate expression of an undesirable gene (such as a viral or tumorigenic gene) in the patient's DNA.

Detailed Description Paragraph Right (57):
Non-viral membrane-associated proteins may also be used to alter the host range of vector particles. Representative examples include polypeptides which act as ligands for given cell surface receptors or other cell surface moieties. Depending on the tissue distribution of the receptor for the protein in question, the retroviral vector could be targeted to a vast range of human cells, to a subset of cells, or to a single cell type. Thus, for example, all human cells, all white blood cells, or only T-helper cells could be targeted.

Detailed Description Paragraph Right (58):
When a ligand to be included within the retroviral envelope is not a naturally occurring membrane-associated protein, it is necessary to associate the ligand with the membrane, preferably by making a "hybrid" or "chimeric" envelope protein. It is important to understand that such hybrid envelope proteins can contain extracellular domains from proteins other than other viral or retroviral env proteins. To accomplish this, the gene coding for the ligand can be functionally combined with sequences coding for a membrane-associated domain. By "naturally occurring membrane associated protein", it is meant those proteins that in their native state exist in vivo in association with lipid membrane such as that found associated with a cell membrane or on a viral envelope. As such, hybrid envelopes can be used to tailor the tropism (and effectively increase titers) of a retroviral vector coding for full length factor VIII, as the extracellular component of env proteins from retroviruses are responsible for specific receptor binding. The cytoplasmic domain of these proteins, on the other hand, play a role in virion formation. The present invention recognizes that numerous hybrid env gene products (i.e., specifically, retroviral env proteins having cytoplasmic regions and extracellular binding regions which do not naturally occur together) can be generated and may alter host range specificity. As a result, recombinant retroviruses can be produced that specifically bind to targeted cells.

Detailed Description Paragraph Right (61):
Vector particles having non-native membrane-associated ligands as described herein, will, advantageously, have a host range determined by the ligand-receptor interaction of the membrane-associated protein. Thus, for targeted delivery of retroviral vectors encoding full length factor VIII, a vector particle having altered host range can be produced using the methods of the present invention. The

ligand will be selected to provide a host range including the targeted cell type. Many different targeting strategies can be employed in connection with this aspect of the invention. For example, there are a number of progenitor cell types found in bone marrow that differentiate into blood cells. Many blood cells have relatively short life spans and therefore progenitor cells must continually divide and differentiate to replace the lost cells. In a preferred embodiment, gene therapy for hemophilia targets hematopoietic progenitor cells, including pluripotent stem cells. These progenitor cells are known to have unique cellular determinants that permit histological identification, separation from other cell types by various techniques, including fluorescence activated cell sorting (FACS) and positive and negative selection [see U.S. Pat. No. 5,061,620], and which can be used as cell receptors for the membrane-associated proteins of the vector particles of the present invention.

Detailed Description Paragraph Right (65):
Targeting a retroviral vector encoding full length factor VIII to a predetermined locus on a chromosome may also be employed. Clear advantages of such targeting include avoidance of insertional mutagenesis and assuring integration at sites known to be transcriptionally active. Techniques for targeting proviral integration to specific sites include integrase modification. See U.S. Ser. No. 08/156,789, supra.

Detailed Description Paragraph Right (71):
Another aspect of the invention concerns the preparation of recombinant retroviral particles. Retroviral particles according to the invention can be produced in a variety of ways, as those in the art will appreciate. For example, producer cells, i.e., cells containing all necessary components for retroviral vector packaging (including a nucleic acid molecule encoding the retroviral vector), can be grown in roller bottles, in bioreactors, in hollow fiber apparatus, and in cell hotels. Cells can be maintained either on a solid support in liquid medium, or gown as suspensions. A wide variety of bioreactor configurations and sizes can be used in the practice of the present invention.

Detailed Description Paragraph Right (82):
Recombinant retroviruses encoding full length factor VIII may also be concentrated by an aqueous two-phase separation method. Briefly, polymeric aqueous two-phase systems may be prepared by dissolving two different non-compatible polymers in water. Many pairs of water-soluble polymers may be utilized in the construction of such two-phase systems, including for example polyethylene glycol ("PEG") or methylcellulose, and dextran or dextran sulfate (see Walter and Johansson, Anal. Biochem. 155:215-242, 1986; Albertsson, "Partition of Cell Particles and Macromolecules" Wiley, N.Y., 1960). As described in more detail below in Example 13, utilizing PEG at concentrations ranging from 5% to 8% (preferably 6.5%), and dextran sulfate at concentrations ranging from 0.4% to 1% (preferably 0.4), an aqueous two-phase system may be established for purifying recombinant retroviruses. Utilizing such procedures, approximate 100-fold concentration can be achieved with yields of approximately 50% or more of the total starting retrovirus.

Detailed Description Paragraph Right (89):
As an alternative to in vivo production, the retroviral packaging proteins may be produced, together or separately, from appropriate cells. However, instead of introducing a nucleic acid molecule enabling production of the viral vector, an in vitro packaging reaction is conducted comprising the gag, pol, and env proteins, the retroviral vector, tRNA, and other necessary components. The resulting retroviral particles can then purified and, if desired, concentrated.

Detailed Description Paragraph Right (90):
Another aspect of the invention relates to pharmaceutical compositions comprising recombinant retroviral vectors as described above, in combination with a pharmaceutically acceptable carder or diluent. Retroviral particles comprising such retroviral vectors can be formulated in crude or, preferably, purified form. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (e.g., lyophilized) which is resuspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or nasal, oral, vaginal, sub-lingual, inhalant, intraocular, enteric, or rectal administration.

Detailed Description Paragraph Right (91):
Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions, preferably

buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin (HSA). A particularly preferred composition comprises a vector or recombinant virus in 10 mg/mL mannitol, 1 mg/mL HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl . In this case, since the recombinant retroviral vector represents approximately 1 .mu.g of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70.degree. C. for at least six months.

Detailed Description Paragraph Right (92):
Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Additionally, such compositions may include inhibitors of complement activation, such as saccharides that compete with pre-existing human antibodies against alpha galactose epitopes, for example B-Disaccharide-R (Chembiomed), B-Disaccharide (Dextra), B-Trisaccharide (Dextra), B-Tetrasaccharide (Dextra), A-Fucosylated trisaccharide-R, 6-0-B-D-Galactopyranosyl-D-galactose, A-Fucosylated trisaccharide-R, Decay Accelarating Factor, and HRF20 [Neethling, et al. (1994), Transplantation, vol. 57, pp:959-963; Hayashi, et al. (1994), Transplantation Proceedings, vol. 26, no. 3, pp: 1243-1244]. Such complement inhibitors may be especially effective when used with recombinant retroviruses that are produced in packaging cell lines derived from a species different from that of the patient to whom the composition is to be administered.

Detailed Description Paragraph Right (93):
Pharmaceutical compositions of the present invention may also additionally include factors which suppress an immune response to the retroviral particles encoding full length factor VIII. In addition, pharmaceutical compositions of the present invention may be placed within containers or kits, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will describe the reagent concentration, as well as within certain embodiments, relative mounts of excipient ingredients or diluents (e.g., water, saline or PBS) which may be necessary to reconstitute the pharmaceutical compositions.

Detailed Description Paragraph Right (110):
In another aspect of the present invention, methods are provided for treating hemophilia A, comprising administering to a warm-blooded animal, particularly a human, a recombinant retrovital vector as described above, such that a therapeutically efficacious amount of factor VIII is produced. As used herein, a "therapeutically effective amount" of factor VIII is an amount that promotes blood coagulation in a patient to an extent greater than that observed when the patient was not treated with factor VIII. A "therapeutically effective amount" of a retroviral vector according to the invention refers to the amount that must be administered to produce a therapeutically effective amount of factor VIII in a particular patient. In a patient suffering from hemophilia, a therapeutically effective amount of a retrovital vector is an amount that elicits production of sufficient factor VIII to produce therapeutically beneficial clotting and will thus generally be determined by each patient's attending physician, although serum levels of about 0.2 ng/mL (about 0.1% of "normal" levels) or more will be therapeutically beneficial. Typical dosages will range from about 10.sup.5 to 10.sup.12 infectious retroviral particles, with dosages of 10.sup.7 to 10.sup.10 infectious particles being preferred. Other dosage measures include the number of International Units of factor VIII detected in the blood of patients treated with retrovital particles according to the invention, as can be measured by an appropriate assay, e.g., a Coatest assay, as described below.

Detailed Description Paragraph Right (111):
In some cases, retroviral vectors according to the invention will be administered as an adjunct to other therapy, such as hormonal, radiation, and/or chemotherapeutic treatment. Factors influencing the amount of full length factor VIII-encoding retroviral particles that will be administered include the age and general condition of the patient, the amount of endogenous, i.e., non-recombinant, factor VIII produced by the patient, etc. Hemophilia A has been categorized into four groups, depending upon serum factor VIII levels, as follows: severe (less than 1% of normal factor VIII levels), moderate, mild, and subclinical (Brinkhous, K. M., Thrombosis Research, 67:329, 1992).

Detailed Description Paragraph Right (112):
In various embodiments of the invention, recombinant retroviral vectors may be administered by various routes in vivo, or ex vivo, as described in greater detail below. Alternatively, the retroviral vectors of the present invention may also be administered to a patient by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner, et al., Proc. Natl. Acad. Sci. USA, 84:7413, 1989), direct DNA injection (Acsadi, et al., Nature, 352:815, 1991; microprojectile bombardment (Williams, et al., Proc. Nat'l. Acad. Sci. USA, 88:2726, 1991); liposomes of several types (see e.g., Wang, et al., Proc. Nat'l. Acad. Sci. USA, 84:7851, 1987); CaPO.sub.4 (Dubensky, et al., Proc. Nat'l. Acad. Sci. USA, 81:7529, 1984); DNA ligand (Wu, et al., J. Biol. Chem., 264:16985, 1989); or administration of nucleic acids alone (WO 90/11092). Other possible methods of administration can include injection of producer cell lines into the blood or, alternatively, into one or more particular tissues, grafting tissue comprising cells transduced with retroviral vectors according to the invention, etc.

Detailed Description Paragraph Right (114):
Transdermal or topical application of a pharmaceutical composition comprising a retroviral vector according to the invention may be used as an alternate route of administration because the skin is the most expansive and readily accessible organ of the human body. Transdermal delivery systems (TDS) are capable of delivering a retroviral particle through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically effective. TDS provide a variety of advantages, including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, a recombinant retrovirus encoding full length factor VIII, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described and include, but are not limited to, gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi, et al., J. Controlled Release, 29:177, 1994). These polymers can be dermatologically formulated into aqueous, powder, or oil phases. Various combinations can produce lotions, pastes ointments, creams, and gels, alone or together with the

Detailed Description Paragraph Right (118):
The nasal cavity also offers an alternative route of administration for compositions comprising a retroviral vector encoding full length factor VIII. For instance, the human nasal cavities have a total surface area of approximately 150 cm.sup.2 and are covered by a highly vascular mucosal layer. A respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells, lines most of the nasal cavity (Chien, et al, Crit. Rev. in Therap. Drug Car. Sys., 4:67, 1987). The subepithelium contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation, avoiding first-pass metabolism in the liver. Thus, delivery to the upper region of the nasal cavity may result in slower clearance and increased bioavallability of retroviral particles. The absence of cilia in this area is an important factor in the increased effectiveness of nasal sprays as compared to drops. The addition of viscosity-building agents, such as methycellulose, etc. can change the pattern of deposition and clearance of intranasal applications. Additionally, bioadhesives can be used as a means to prolong residence time in the nasal cavity. Various formulations comprising sprays, drops, and powders, with or without the addition of absorptive enhancers, have been described (see Wearley, L, supra).

Detailed Description Paragraph Right (120):
The human rectum has a surface area of between 200 to 400 cm.sup.2 and is abundant in blood and lymphatic vessels. This offers an alternative route for administrating compositions according to the invention. Depending on the actual site of administration, it may be possible to bypass first-pass metabolism by the liver. Targeting of the systemic circulation can be achieved by delivering the vehicle to an area behind the internal rectal sphincter which allows absorption directly into the inferior vena cava, thereby bypassing the portal circulation and avoiding metabolism in the liver. The liver can be targeted by delivering the vehicle to the region of the ampulla recti, which allows absorption into the portal system (Ritschel, supra.). Interestingly, liver transplantation rectifies hemophilia A, and factor VIII mRNA is detectable in the liver and in isolated hepatocytes (Zatloukal, et al., supra). These results suggest that delivery of retroviral vectors as described herein to the liver, directly or indirectly, will be among those that are preferred in the practice of this invention.

Detailed Description Paragraph Right (123):
As an alternative to in vivo adminstration of the retroviral vectors and particles of the invention, ex vivo adminstration can be employed. Ex vivo treatment envisions withdrawl or removal of a population of cells from a patient. Exemplary cell populations include bone marrow cells, liver cells, and blood cells from the umbilical cord of a newborn. Such cells may be be processed to purify desired cells for transduction prior to such procedures, for instance to obtain subsets of such cell populations, e.g., CD34.sup.+ bone marrow progenitor cells. Preferred methods of purification include various cell sorting techniques, such as antibody panning, FACS, and affinity chromatography using a matrix coupled to antibodies specifcially reactive to the desired cell type(s). Isolated cells are then transduced, after which they may be immediately re-introduced to the patient from which they were withdrawn. Alternatively, the cells may be expanded in culture by various techniques known to those skilled in the art prior to re-introduction.

Detailed Description Paragraph Right (124):
In another embodiment of the invention, retroviral vectors encoding full length factor VIII are administered to hemophilic patients in conjunction with another therapeutic compound. As those in the art will appreciate, such compounds may include, but are not limited to, other gene delivery vehicles designed to deliver one or more other therapeutic genes to the patient, as is described in U.S. Ser. No. 08/368,210, filed on a date even herewith). For instance, a patient suffering from hemophilia A may also be infected with HIV and/or HBV. Thus, such a patient may also be treated with a gene delivery vehicle(s) designed to treat such a disease(s), for instance by stimulating the patient's immune system [see U.S. Ser. No. 08/136,739, supra; see also U.S. Ser. No. 08/032,385, filed Mar. 17, 1993]or by conditioning infected cells to become sensitive to a cytotoxic compound to be administered later [see U.S. Ser. No. 08/155,944, filed Nov. 18, 1993].

Detailed Description Paragraph Right (126):
This example describes the construction of several retroviral vectors comprising a nucleic acid molecule encoding a full length factor VIII polypeptide. As will be clear to those in the art, other comparable retroviral vectors can be similarly constructed.

Detailed Description Paragraph Right (128):
A 1.0 kb MoMLV 3' LTR Eco RI-Eco RI fragment from N2 is next cloned into plasmid SK.sup.+, resulting in a construct designated N2R3.sup.-. A 1.0 kb Cla I-Hind III fragment is then purified from this construct. The Cla I--Cla I dominant selectable marker gene fragment from the pAFVXM retroviral vector (Kriegler et al., Cell 38:483, 1984; St. Louis et al., (1988) Proc. Nat'l, Acad. Sci. USA, vol. 85, pp:3150-3154), comprising a SV40 early promoter driving expression of the neomycin (neo) phosphotransferase gene, is cloned into the SK.sup.+ plasmid. This construct is designated SK.sup.+ SV.sub.2 -neo. A 1.3 kb Cla I-Bst BI gene fragment is then purified from the SK.sup.+ SV.sub.2 -neo plasmid.

Detailed Description Paragraph Right (129):
A plasmid encoding the KT-3 (pKT-3) retroviral vector is generated by ligating the 1.0 kb MoMLV 3' LTR Cla I-Hind III fragment (from N2R3.sup.-) into like-digested pUC31/N2R5gM. The 1.3 Kb Cla I-Bst BI fragment encoding the neo gene is then inserted into the Cla site of the resultant plasmid construct.

Detailed Description Paragraph Right (130):
A plasmid, pKT-1, is also constructed encoding a retrovital backbone similar to KT-3, with the exception that the dominant selectable marker gene, neo, is not inserted into the plasmid. pKT-1 is used to produce KT-1-based retrovital vectors comprising a full length factor VIII gene.

Detailed Description Paragraph Right (131):
The following is a description of the construction of several retroviral vectors encoding a full-length factor VIII cDNA. Due to the packaging constraints of retroviral vectors and because selection for transduced cells is not a requirement for therapy, a retroviral backbone, e.g., KT-1, lacking a selectable marker gene is employed.

Detailed Description Paragraph Right (132):
A gene encoding full length factor VIII can be obtained from a variety of sources. One such source is the plasmid pCIS-F8 (EP 0 260 148 A2, published Mar. 3, 1993),

which contains a full length factor VIII cDNA whose expression is under the control of a CMV major immediate-early (CMV MIE) promoter and enhancer. The factor VIII cDNA contains approximately 80 bp of 5' untranslated sequence from the factor VIII gene and a 3' untranslated region of about 500 bp. In addition, between the CMV promoter and the factor VIII sequence lies a CMV intron sequence, or "cis" element. The cis element, spanning about 280 bp, comprises a splice donor site from the CMV major immediate-early promoter about 140 bp upstream of a splice acceptor from an immunoglobulin gene, with the intervening region being supplied by an Ig variable region intron. The sequence of this region, from splice donor to splicew acceptor, is presented in SEQ ID NO: 3.

Detailed Description Paragraph Right (133):
i. Construction of a Plasmid Encoding Retroviral Vector JW-2.

Detailed Description Paragraph Right (134):
A plasmid, pJW-2, encoding a retroviral vector for expressing full length factor VIII is constructed using the KT-1 backbone from pKT-1. To facilitate directional cloning of the factor VIII cDNA insert into pKT-1, the unique Xho I site is convened to a Not I site by site directed mutagenesis. The resultant plasmid vector is then opened with Not I and Cla I. pCIS-F8 is digested to completion with Cla I and Eag I, for which there are two sites, to release the fragment encoding full length factor VIII. This fragment is then ligated into the Not I/Cla I restricted vector to generate a plasmid designated pJW-2.

Detailed Description Paragraph Right (135):
ii. Construction of a Plasmid Encoding Retroviral Vector ND-5.

Detailed Description Paragraph Right (136):
A plasmid vector encoding a truncation of about 80% (approximately 370 bp) of the 3' untranslated region of the factor VIII cDNA, designated pND-5, is constructed in a pKT-1 vector as follows: As described for pJW-2, the pKT-1 vector employed has its Xho I restriction site replaced by that for Not I. The factor VIII insert is generated by digesting pCIS-F8 with Cla I and Xba I, the latter enzyme cutting 5' of the factor VIII stop codon. The approximately 7 kb fragment containing all but the 3' coding region of the factor VIII gene is then purified. pCIS-F8 is also digested with Xba I and Pst I to release a 121 bp fragment containing the gene's termination codon. This fragment is also purified and then ligated in a three way ligation with the larger fragment encoding the rest of the factor VIII gene and Cla I/Pst I restricted BLUESCRIPT.RTM. KS.sup.+ plasmid (Stratagene, supra) to produce a plasmid designated pND-2.

Detailed Description Paragraph Right (138):
The factor VIII sequence in pND-3, bounded by Cla I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pJW-1 [a pKT-1 derivative by cutting at the Xho I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)], which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and Cla I and a 3.1 kb fragment is isolated. The two fragments containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce a plasmid designated pND-5.

Detailed Description Paragraph Right (139):
As those in the art will appreciate, after construction of plasmids encoding retroviral vectors such as those described above, such plasmids can then be used in the production of various cell lines from which infectious recombinant retroviruses can be produced. The production of such cell lines is described in the following example.

Detailed Description Paragraph Right (140):
In this example, procedures are described for making packaging and producer cell lines which can make recombinant retroviral particles coding for full length factor VIII. Specifically, production of three packaging cell lines, DA (an amphotropic packaging cell line derived from the canine cell line D 17), HX (a xenotropic packaging cell line derived from the human cell line HT1080), and their packaging intermediates is described below.

Detailed Description Paragraph Right (145):

Highest titers are obtained when retroviral vectors are introduced into packaging cell lines by infection, as opposed to transfection (Miller, et al., Somat. Cell Mol. Genet., 12:175, 1986). Although amphotropic MLV vectors are known to infect these host cell types, the packaging cell lines DA and HA are blocked for infection by amphotropic vectors since they express an amphotropic env protein (i.e., "viral interference"). To overcome the problem of "viral interference," whereby cell lines expressing an amphotropic envelope protein block later infection by amphotropic MLV vectors able to otherwise infect those cell types, vector particles containing other viral envelopes (such as xenotropic env or VSV G protein, which bind to cell receptors other than the amphotropic receptor) may be generated in the following manner. 10 .mu.g of the plasmid DNA encoding the retroviral vector to be packaged is co-transfected into a cell line which expresses high levels of gag/pol with 10 .mu.g of DNA from which either xenotropic env or a VSV G protein is expressed. The resultant vector, containing xenotropic env or VSV G protein, respectively, is produced transiently in the co-transfected cells. Two days after transfection, cell free supernatants are added to prospective packaging cell lines (which express gag, pol, and env). Both types of vector efficiently infect the cells blocked for infection by amphotropic retrovirus. Cell free supematants are then collected from the confluent monolayers and titered by PCR. Cell clones producing the highest liters are selected as packaging cell lines and are referred to as DA (D17 expressing an amphotropic env) and HA (HT1080 expressing an amphotropic env) cells.

Detailed Description Paragraph Right (147):
Again, to avoid viral interference during production of a xenotropic HT1080 producer cell line, i.e., that produces infectious retroviral particles encoding full length factor VIII, "G-hopping" as described above can be employed. 10 .mu.g of the plasmid DNA encoding the retroviral vector to be packaged, e.g., pJW-2 or pND-5, is co-transfected into a cell line which expresses high levels of gag/pol with 10 .mu.g of DNA from which VSV G protein is expressed. Recombinant retroviral particles are produced transiently. Two days after transfection, cell free supernatants are added to prospective HT1080 packaging cell lines which express gag, pol, and xenotropic env. Cell free supernatants are then collected from the confluent monolayers and titered by PCR. Cell clones producing the highest titers are selected as packaging cell lines and are referred to as HX (HT1080 expressing a xenotropic env) cells.

Detailed Description Paragraph Right (149):
As described above, retroviral vector particles containing VSV G protein are made by using 10 .mu.g of plasmid DNA encoding the retroviral vector to be packaged, e.g., pJW-2 or pND-5, is co-transfected with 10 .mu.g of DNA from which VSV G protein is expressed into a cell line which expresses high levels of gag/pol. Cell free supernatants from that culture are used to transduce HT1080 clones expressing relatively high levels of both gag/pol and polytropic env. Cell free supernatants are collected from the confluent monolayers and titered as described above. Clones expressing relatively high levels of both gag/pol and polytropic env are identified, retained, and designated "HP" (HT1080 expressing a polytropic env).

Detailed Description Paragraph Right (150):
The propensity of the packaging cells described above to generate replication competent retrovirus is stringently tested by co-cultivating HX and DA packaging cells containing the vector N2. Since amphotropic vector can infect cells making the xenotropic envelope and vice versa, continuous cross-infection can occur, thereby increasing the probability of generating RCR. RCR is detected by assaying for the production of amphotropic and xenotropic retroviruses, as judged by a vector rescue assay on 293 or Mus dunni cells (NIH NIAID Bethesda, Md.), both of which can detect amphotropic and xenotropic retroviruses.

Detailed Description Paragraph Right (153):
Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 .mu.m filter to remove any cells. On day 1, Mv1Lu cells are seeded at 1.0.times.10.sup.5 cells per well (one well per sample to be tested) on a 6 well plate in 2 mL Dulbecco's Modified Eagle Medium (DMEM), 10% FBS and 8 .mu.g/mL polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37.degree. C., 10% CO.sub.2. On day 2, 1.0 mL of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 mL of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 mL media) of MA virus (Miller, et al., Molec. and Cell Biol., 5:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated

and 3.0 mL of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 mL DMEM and 10% FBS is added to the cells. In addition, the MiCl.sub.1 cells are seeded at 1.0.times.10.sup.5 cells per well in 2.0 mL DMEM, 10% FBS and 8 .mu./mL polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the MiCl.sub.1 cells and incubated overnight at 37.degree. C., 10% CO.sub.2. On day 15, the media is aspirated and 3.0 mL of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the MiCl.sub.1 cells. Using these procedures, it can be shown that full length factor VIII producer cell lines are not contaminated with replication competent retroviruses.

Detailed Description Paragraph Right (156):
The MdH marker rescue cell line is cloned from a pool of Mus dunni cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer, et al., Proc. Nat'l. Acad. Sci. USA, 84:1055, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One mL of test sample is added to a well of a 6-well plate containing 1.times.10.sup.5 MdH cells in 2 mL standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 .mu.g/mL polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 .mu.m cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0.times.10.sup.4 Mus dunni target cells in 2 mL standard culture medium containing polybrene. After 24 hours, supernatants are replaced with standard culture media containing 250 .mu.g/mL of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 .mu.g/mL of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection, by staining with 0.2% Coomassie blue.

Detailed Description Paragraph Right (157):
The production of JW-2 and ND-5 recombinant retroviral particles encoding full length factor VIII from the human xenotropic and canine amphotropic packaging cell lines HX and DA, respectively, are described below.

Detailed Description Paragraph Right (159):
DA packaging cells are seeded at 1.0.times.10.sup.5 cells/3 cm tissue culture dish in 2 mL DMEM and 10% FBS, 4 .mu.g/mL polybrene (Sigma, St. Louis, Mo.) on day 1. On day 2, 3.0 mL, 1.0 mL and 0.2 mL of each of the freshly collected JW-2 or ND-5 retrovirus-containing HX supernatants are added to the cells. The cells are incubated overnight at 37.degree. C. On day 3, the pools of cells are cloned by limiting dilution by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/mL and adding 0.1 mL to each well (1 cell/well) of a 96 well plate (Corning, Corning, N.Y.). Cells are incubated for 14 days at 37.degree. C., 10% CO.sub.2. Twenty-four clones producing JW-2 and 24 clones producing ND-5 are selected and expanded up to 24 well plates, 6 well plates, and finally to 10 cm plates, at which time the clones are assayed for expression of the appropriate retroviral vector and the supernatants are collected and assayed for retroviral liter.

Detailed Description Paragraph Right (160):
The packaging cell line HX may also be transduced with either JW-2 or ND-5 recombinant retroviral vectors generated from a DA producer cell line in the same manner as described for transduction of the DA cells from the HX supernatants.

Detailed Description Paragraph Right (161):
Using the procedures above, DA and HX cell lines are derived that produce either JW-2 or ND-5 retroviral vectors with titers greater than or equal to 1.times.10.sup.6 cfu/mL in culture.

Detailed Description Paragraph Right (162):
As recombinant retroviral vectors encoding full length factor VIII, e.g., JW-2 and ND-5, do not include a gene coding for a selectable marker, titering assays other than those based on selection of drug resistant colonies are required. To this end, antibody and PCR assays, the latter of which is described below, may be employed to determine retroviral vector titer, i.e., the number of infectious particles

comprising the retroviral vectors of the invention. To use PCR to amplify sequences unique to the retroviral vectors of the invention, various primers are required. Such primers can readily be designed by those skilled in the art and will depend on the retroviral vector backbone employed and the components thereof, the particular region(s) desired to be amplified, etc. Representative examples of particular primer pairs include those specific for LTR sequences, packaging signal sequences or other regions of the retroviral backbone, and also include primers specific for the full length factor VIII gene in the vector, which, due to its derivation from cDNA, lacks intron sequences likely to be present in endogenous factor VIII genomic sequences. Additional advantages in using such a PCR titering assay include the ability to assay for genome rearrangement, etc. As those in the art will appreciate, the PCR titering assay described below will also be applicable to gene transfer systems other than retroviral systems. For instance, it can be used to determine titers for gene transfer systems derived from adenoviruses, pox viruses, alphaviruses, direct or "naked" DNA, etc.

Detailed Description Paragraph Right (163):
In the practice of the present invention, the PCR titering assay is performed by growing a known number of HT1080 cells, typically 1.times.10.sup.5 cells, transduced with a retroviral vector capable of directing full length factor VIII expression on 6-well plates for at least 16 hr. before harvest. The retroviral vectors used for these transductions are obtained from either cell culture supernatants or blood. One well per plate is reserved for cell counting. Cells from the other wells are lysed and their contents isolated. DNA is prepared using a QIAmp Blood Kit for blood and cell culture PCR (QIAGEN, Inc., Chatsworth, Calif.). DNAs are resuspended at 5.times.10.sup.6 cell equivalents/mL, where one cell equivalent is equal to the DNA content of one cell.

Detailed Description Paragraph Right (164):
To calculate titer, a standard curve is generated using DNA isolated from untransduced HT1080 cells (negative control) and HT1080 cells transduced with a known vector and having one copy of that vector per cell genome (positive control), such as may be prepared from packaging cell lines transduced with a retroviral vector encoding a selectable marker, e.g., neomycin resistance. For both the positive and negative controls, DNA is resuspended at 5.times.10.sup.6 cell equivalents/mL. The standard curve is generated by combining different amounts of the positive and negative control DNA, while keeping the total amount of DNA constant, and amplifying specific sequences therefrom by PCR using primers specific to a particular region of the retroviral vector. A representative group of mixtures for generating a standard curve is:

Detailed Description Paragraph Right (165):
In some situations it may be desirable to avoid using more than one cell line in the process of generating producer lines. For example, DA cells are seeded at 5.0.times.10.sup.5 cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% irradiated (2.5 megarads minimum) FBS. On day 2, the media is replaced with 5.0 mL fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 60 .mu.l 2.0M CaCl.sub.2, 10 lag of a plasmid from which VSV G will be expressed, 10 .mu.g pND-5 retroviral vector plasmid, and deionized water to a volume of 400 pl. The DNA-CaCl.sub.12 solution is then added dropwise with constant agitation to 400 .mu.l of 2X precipitation buffer (50 mM HEPES-NaOH, pH 7.1, 0.25M NaCl and 1.5 mM Na.sub.2 HPO.sub.4 -NaH.sub.2 PO.sub.4). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of DA cells plated the previous day. The cells are incubated with the DNA precipitate overnight at 37.degree. C. On day 3, the medium is removed and fresh medium is added. The supernatant containing G-pseudotyped virus is removed on day 4, passed through a 0.45 .mu.m filter and used to infect DA packaging cells as follows.

Detailed Description Paragraph Right (166):
DA cells are seeded at 5.0.times.10.sub.5 cells on a 10 cm tissue culture dish in 10 mL DMEM and 10% FBS, 4 mg/mL polybrene (Sigma, St. Louis, Mo.) on day 1. On day 2, 2.0 mL, 1.0 mL or 0.5 mL of the freshly collected and filtered G-pseudotyped retrovirus-containing supernatant is added to the cells. The cells are incubated with the retrovirus overnight at 37.degree. C. Because no selectable marker is carded on the retroviral vector, no selection step is employed. Instead, cell pools are tested for expression and then dilution cloned by removing the cells from the plate, counting the cell suspension, diluting the cell suspension down to 10 cells/mL and adding 0.1 mL to each well (1 cell/well) of a 96-well plate. Cells are

incubated for 2 weeks at 37.degree. C., 10% CO.sub.2. Numerous clones are selected and expanded up to 24-well plates, then 6-well plates, and finally 10 cm plates, at which time the clones are assayed for expression and the supernatants are collected and assayed for retroviral titer as described above.

Detailed Description Paragraph Right (167):
In order to test the ability of retroviral vectors made in accordance with the teachings herein to transfer factor VIII expression, cells must be transduced with such vectors and the media or, in the case of therapeutic treatment, blood must be analyzed for the amount of factor VIII produced. Cell lines or patient cells transduced with retroviral vectors according to the invention are examined for expression of factor VIII by Coatest factor VIII:C analysis or by standard clotting assay.

Detailed Description Paragraph Right (169):
The coagulation cascade is triggered by activation of factor X (which becomes factor Xa) by factor IXa in the presence of calcium and phospholipids, and is greatly enhanced by factor VIII, which acts as a co-factor. By using an in vitro assay (COATEST.RTM., Chromogenix AB, Monlndal, Sweden) where optimal amounts of calcium and phospholipids and an excess of factors IXa and X, the rate of activation of factor X depends solely on the amount of factor VIII. Factor Xa is known to hydrolyze the chromogenic substrate S-2222 (Bz-Ile-Glu(.gamma.-OR)-Gly-Arg-pNA), releasing pNA which can be detected spectrophotometrically at 405 nm. Signal intensity is proportional to factor VIII activity. Using such an assay, the amount of factor VIII produced either in tissue culture or in a patient can be determined. One International Unit (IU) of factor VIII activity is that amount of activity measured in 1.0 mL of pooled normal human plasma. The assay is performed as follows:

Detailed Description Paragraph Right (170):
Cell free media containing factor VIII is obtained. For patient samples, 9 volumes of blood is mixed with one volume of 0.1M sodium titrate, pH 7.5, and centrifuged at 2,000.times.g 5-20 min. at 20.degree.-25.degree. C. to pellet cells. Due to heat lability of factor VIII, plasma samples should be tested within 30 min. of isolation or stored immediately at -70.degree. C, although as much as 20% of factor VIII activity may be lost during freezing and thawing. When culture media is assayed, cells are similarly removed by centrifugation and an equal volume of working buffer (Coatest Kit).

Detailed Description Paragraph Right (171):
As discussed above, serum levels of factor VIII in non-hemophilic patients are in the range of 200 ng/mL. Depending upon the range of factor VIII expected, either above or below 20% of normal, either of the two procedures below are used. In either case, a standard curve based on dilutions of normal human plasma (1.0 IU factor VIII/mL) is used and the assays should be performed in plastic tubes. When factor VIII levels are expected to be 20% or more of normal, a solution is prepared containing one volume of phospholipid emulsified from porcine brain and 5 volumes of reconstituted, lyophilized factor IX and factor X prepared as described by the manufacturer. This solution is stored at 2.degree.-8.degree. C. In an adaptation of the Coatest assay procedure for use on 96 well Falcon plates, 40 .mu.L of this solution is mixed with 20 .mu.L of plasma plus 20 .mu.L of working buffer. The mixture is incubated at 37.degree. C. for 4-5 min., after which 20 .mu.L of a 0.025M CaCl.sub.2 stock solution is added, followed by a 5 min. 37.degree. C. incubation. 40 .mu.L of the chromogenic reagent (20 mg S-2222, 335 .mu.g synthetic thrombin inhibitor, I-2581, in 10 mL) is then mixed in. After a 5 min. incubation at 37.degree. C., 20 .mu.L of 20% acetic acid or 2% citric acid is added to stop the reaction. Absorbance is then measured against a blank comprising 50 mM Tris, pH 7.3, and 0.2% bovine serum albumin (BSA).

Detailed Description Paragraph Right (175):
1.0.times.10.sub.4 HT1080 cells are seeded into each well of a 6 well plate containing 2 mL of DMEM, 10% FBS, and 4 mg/mL polybrene. The next day, 1-2 mL of supernatant obtained from HX cells transfected with pJW-2 is added to each well. After the cells become confluent, media is harvested from each well and subjected to a Coatest assay. These results, when correlated with those of a standard curve generated using dilutions of pooled normal human plasma, indicate that the HT1080 cells transduced with HX/JW-2 secrete about 30 ng/day/10.sup.6 cells of factor VIII into the media.

Detailed Description Paragraph Right (177):
Experiments similar to those for HX/JW-2 but using HT1080 cells transduced with retroviral vectors produced from a dilution cloned HX/ND-5 producer cell line and having a PCR-determined titer of 1.2.times.10.sup.4 vectors/mL reveal that factor VIII is produced and secreted in transduced HT1080 cells at a level of at least 5 times that observed for HX/JW-2.

Detailed Description Paragraph Right (179):
Transfer of expression in primary human fibroblasts obtained from a skin punch biopsy taken from the forearm of a human volunteer is conducted by seeding approximately 3.times.10.sup.4 primary human fibroblasts in each well of a 6 well plate. The cells are grown in 2 mL/well of Modified Eagle's Minimal Media (Irvine Scientific, Santa Ana, Calif.) containing 15% FBS and 200 mM L-glutamine. The day after seeding, various amounts of supernatant (44 .mu.L, 133 .mu.L, and 400 .mu.L) obtained from DA cells transfected with a VSV G-encoding expression vector and pJW-2 diluted to a total volume of 1-2 mL is added to each well. After the cells become confluent (normally 3-6 days post-infection), media is harvested from each well and subjected to a Coatest assay. The level of factor VIII expressed from these cells, as measured by Coatest assay, are shown in FIG. 4.

Detailed Description Paragraph Right (180):
Crude recombinant retroviral particles encoding full length factor VIII are obtained from a Celligan bioreactor (New Brunswick, New Brunswick, N.J.) containing DA or HX cells transduced with a recombinant retroviral vector according to the invention bound to the beads of the bioreactor matrix. The cells release the recombinant retroviral particles into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 .mu.m filter and then through a 0.65 .mu.m filter to clarify the supernatant. This retroviral particle-containing filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, Mass.). Approximately 50 units of DNase (Intergen, New York, N.Y.) per mL of concentrate is added to digest exogenous DNA. The digest is diafiltrated in the same cross flow system against 150 mM NaCl mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, N.J.), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant retrovirus is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

Detailed Description Paragraph Left (2):
B. Production of Plasmid Vectors Encoding Full-Length Factor VIII

Detailed Description Paragraph Left (10):
5.0 .mu.L from each tube is placed into one of eight reaction tubes (duplicates are also prepared), with the remainder being stored at -20.degree. C. 5.0 .mu.L from each sample DNA preparation are placed into their own reaction tubes in duplicate. PCR reactions (50 .mu.L total volume) are then initiated by adding 45.0 .mu.L of a reaction mix containing the following components per tube to be tested: 24.5 .mu.L water, 5 .mu.L 10X reaction PCR buffer, 4 .mu.L of 25 mM MgCl.sub.2, 4 .mu.L dNTPs (containing 2.5 mM of each of dATP, dGTP, dCTP, and dTTP), 5 .mu.L of primer mix (100 ng or each primer), 0.25 .mu.L TaqStart monoclonal antibody (Clontech Laboratories, Inc., Palo Alto, Calif.), 1.00 .mu.L TaqStart buffer (Clontech Labs, Inc.), and 0.25 .mu.L AmpliTaq DNA polymerase (Perkin-Elmer, Inc., Norwalk, Conn.). Just prior to aliquoting the reaction mix to the reaction tubes, 1 .mu.L of .alpha.-.sup.32 P dCTP (250 .mu.Ci; 3000 C/mmol, 10 mCi/mL, Amersham Corp., Arlington Heights, Ill.) is added into the reaction mix. After aliquoting 45.0 .mu.L the reaction mix into each of the reaction tubes, the tubes are capped and placed into a thermocycler. The particular denaturation, annealing, elongation times and temperatures, and number of thermocycles will vary depending on size and nucleotide composition of the primer pair used. 20-25 amplification thermocycles are then performed. 5 .mu.L of each reaction is then spotted on DE81 ion exchange chromatography paper (Whatman, Maidstone, England) and air dried for 10 min. The filter is then washed five times, 100 mL per wash, in 50 mM Na.sub.2 PO.sub.4, pH 7, 200 mM NaCl , after which it is air dried and then sandwiched in Saran Wrap. Quantitation is performed on a PhosphoImager SI (Molecular Dynamics, Sunnyvale, Calif.). Filters are typically exposed to a phosphor screen, which stores energy from ionizing radiation, for a suitable period, typically about 120 min. After exposure, the phosphor screen is scanned, whereby light is emitted in proportion to the radioactivity on the original filter. The scanning results are then downloaded and plotted on a log scale as cpm (ordinate) versus percent positive control DNA (abscissa). Titers (infectious units/mL) for each sample are calculated by

multiplying the number of cells from which DNA was isolated by the percentage (converted to decimal form) determined from the standard curve based on the detected radioactivity, divided by the volume of retroviral vector used to transduce the cells. As will be appreciated by those in the art, other methods of detection, such as colorimetric methods, may be employed to label the amplified products.

Detailed Description Paragraph Left (12):
E. Reuroviral Vector-Mediated Transfer of Factor VIII Expression.

Detailed Description Paragraph Center (3):
Generation of Recombinant Retroviral Vectors

Detailed Description Paragraph Center (11):
Construction of Retroviral Vectors Comprising a Full Length Factor VIII Gene

Detailed Description Paragraph Center (13):
Production of Cell Lines to Make Retroviral Vector Particles Comprising a Full Length Factor VIII Gene

Detailed Description Paragraph Center (15):
Production of Retroviral Vector Particles Encoding Full Length Factor VIII

Detailed Description Paragraph Center (17):
Production of Retroviral Vector Particles Encoding Full Length Factor VIII

CLAIMS:

1. A retrovital vector comprising gene expression control elements operably linked to a nucleotide sequence encoding a full-length factor VIII polypeptide.

2. A retroviral vector according to claim 1 wherein the retrovirus is selected from the group consisting of MoMLV, GALV, FeLV, and FIV.

3. A retroviral vector according to claim 1 wherein said gene expression control elements comprise a viral promoter.

7. A recombinant retroviral particle comprising the retroviral vector of claim 1 in association with structural proteins.

8. A retrovital vector comprising gene expression control elements operably linked to a nucleotide sequence encoding a full-length factor VIII polypeptide, wherein the full-length factor VIII polypeptide is encoded by a nucleic acid molecule selected from the group consisting of:

(a) a nucleotide sequence set forth in SEQ ID NO: 1, except that a uracil ("U") replaces every thymine ("T");

(b) a nucleotide sequence which would hybridize under stringent conditions to the complementary nucleotide sequence of (a); and

(c) a nucleotide sequence which, but for the degeneracy of the genetic code, would hybridize to the nucleotide sequences of (a) or (b).

9. A retrovital vector according to claim 8 wherein the retrovirus is selected from the group consisting of MoMLV, GALV, FeLV, and FIV.

10. A retroviral vector according to claim 8 wherein said gene expression control elements comprise a viral promoter.

11. A retroviral vector according to claim 10 wherein said vital promoter is selected from the group consisting of a retrovital LTR promoter, a SV40 promoter, a CMV MIF promoter, and a MPMV promoter.

13. An ex vivo producer cell comprising a nucleic acid construct which expresses retrovital structural proteins, and also comprising the retrovital vector according to claim 8, wherein said producer cell packages said retroviral vector in association with said packaging structural proteins to produce recombinant retroviral particles.

14. A recombinant retrovital particle comprising the retroviral vector of claim 8 and in association with retrovital structural proteins.

L3: Entry 1 of 6 File: USPT Dec 11, 2001

DOCUMENT-IDENTIFIER: US 6329199 B1
TITLE: Retroviral vectors produced by producer cell lines resistant to lysis by human serum

Abstract Paragraph Left (1):
Retroviral vectors which are resistant to inactivation by human serum. The retroviral vectors are produced in a cell line which is resistant to lysis by human serum, such cell lines including the HOS, Mv-1-Lu, HT1080, TE671, and human 293 cell lines, as well as cell lines derived therefrom. Such retroviral vectors are especially useful as in vivo gene delivery vehicles.

Brief Summary Paragraph Right (1):
This invention relates to retroviral vectors which are resistant to inactivation by human serum. More particularly, this application relates to retroviral vectors generated from cells which are resistant to lysis by human serum. In another aspect, this invention relates to gene therapy using such vectors.

Drawing Description Paragraph Right (11):
FIG. 10 is a graph of titers of retroviral vectors having a VSV-G retroviral envelope wherein said viruses were produced by a gp7C-derived mouse cell line, upon exposure to human serum or inactivated fetal bovine serum;

Drawing Description Paragraph Right (12):
FIG. 11 is a graph of titers of retroviral vectors produced by the CAK8 cell line, upon exposure to human serum or inactivated fetal bovine serum;

Drawing Description Paragraph Right (13):
FIG. 12 is a graph of the titers of retroviral vectors produced by the PA317 cell line, upon exposure to human serum or inactivated fetal bovine serum;

Drawing Description Paragraph Right (14):
FIG. 13 is a graph of the titers of retroviral vectors produced by the CAK8 cell line, upon exposure to human serum for periods of time up to 180 minutes; and

Drawing Description Paragraph Right (15):
FIG. 14 is a graph of the titers of retroviral vectors produced by the PA317 cell line, upon exposure to human serum for periods of time up to 180 minutes.

Detailed Description Paragraph Right (1):
Applicants have discovered that when retroviral vectors are produced from a cell line which is resistant to lysis by human serum, such retroviral vectors may be resistant to complement inactivation by human serum, and that such complement resistance is not dependent necessarily upon the envelope employed, even when the envelope is from a murine Type C amphotropic retrovirus.

Detailed Description Paragraph Right (2):
Thus, the present invention is directed to retroviral vectors which have been produced by a cell line which is resistant to lysis by human serum. The present invention also is directed to gene therapy employing such retroviral vectors, wherein such retroviral vectors contain at least one polynucleotide encoding a therapeutic agent.

Detailed Description Paragraph Right (3):
In accordance with an aspect of the present invention, there is provided a retroviral vector resistant to inactivation by human serum. The retroviral vector

ha's been produced in a cell line which is resistant to lysis by human serum.

Detailed Description Paragraph Right (5):
The term "cell line derived from the HOS, TE671, HT1080, Mv-1-Lu, or human 293 cell lines," as used herein, means a cell line formed by transfecting one of the above-mentioned cell lines with one or more expression vehicles (e.g., plasmid vectors or retroviral vectors or retroviral vector genomes) including polynucleotides encoding various gag, pol, and env proteins. The gag and pol retroviral proteins may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, Rous Sarcoma Virus, RD114, BaEV, GALV, SSAV, FeLV-B, human immunodeficiency virus, and avian leukosis virus. Alternatively, the gag/pol proteins may be modified or chimeric gag/pol constructs. The envelope may be an amphotropic envelope, an ecotropic envelope, or a xenotropic envelope, or may be an envelope including amphotropic and ecotropic portions. The envelope may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, Rous Sarcoma Virus, RD114, BaEV, GALV, SSAV, FeLV-B, amphotropic murine leukemia viruses (MLV-A), human immunodeficiency virus, avian leukosis virus and NZB virus. Alternatively, the env proteins may be modified or chimeric env constructs, or may be obtained from non-retroviruses, such as vesicular stomatitis virus and HVJ virus. Such cells also may include other polynucleotides such as, for example, polynucleotides encoding selectable markers.

Detailed Description Paragraph Right (7):
The retroviral vectors, in one embodiment, may be produced by transfecting the cells with a retroviral plasmid vector as described hereinbelow. Alternatively, the retroviral vectors may be produced by infecting the cells with a retroviral vector from another packaging cell. The cells also are provided with the retroviral packaging function. The packaging function may be provided by a replication competent retrovirus, or may be provided by a transient system which includes one or more expression vehicles (e.g., plasmid vectors) including polynucleotides encoding the gag, pol, and env proteins. Such functions also may be provided by transfecting stably the cell line with one or more expression vehicles (e.g., plasmid vectors) including polynucleotides encoding the gag, pol, and env proteins.

Detailed Description Paragraph Right (9):
As used herein, the term "retroviral plasmid vector" means a plasmid which includes all or part of a retroviral genome including 5' and 3' retroviral long-term repeat (LTR) sequences, a packaging signal (.psi.), and may include one or more polynucleotides encoding a protein(s) or polypeptide(s) of interest, such as a therapeutic agent or a selectable marker. The term "therapeutic" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents.

Detailed Description Paragraph Right (10):
In one embodiment, the retroviral plasmid vector may be derived from Moloney Murine Leukemia Virus and is of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al., J. Virol., Vol. 61, pgs. 1639-1649 (1987) and Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragments or truncations thereof, are not expressed.

Detailed Description Paragraph Right (11):
In another preferred embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus and includes at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a most preferred embodiment, the retroviral plasmid vector includes each of these cloning sites. Such vectors are further described in U.S. patent application Ser. No. 08/340,805, filed Nov. 17, 1994, now U.S. Pat. 5,672,510, and in PCT Application No. W091/10728, published Jul. 25, 1991, and incorporated herein by reference in their entireties.

Detailed Description Paragraph Right (12):
When a retroviral plasmid vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites

which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral plasmid vector. The shuttle cloning vector also includes at least one desired polynucleotide encoding a therapeutic agent which is capable of being transferred from the shuttle cloning vector to the retroviral plasmid vector.

Detailed Description Paragraph Right (15):
The retroviral plasmid vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and .beta.-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

Detailed Description Paragraph Right (16):
Other retroviral plasmid vectors which may be employed include, but are not limited to, retroviral plasmid vectors derived from Human Immunodeficiency Virus, Rous Sarcoma Virus, avian leukosis virus, NZB virus, the feline endogenous virus RD114, feline leukemia virus B (FeLV-B), simian sarcoma associated virus (SSAV), baboon endogenous virus (BaEV), and gibbon ape leukemia virus (GALV). It is to be understood, however, that the scope of the present invention is not to be limited to any particular retroviral plasmid vector.

Detailed Description Paragraph Right (17):
Polynucleotides encoding therapeutic agents which may be contained in the retroviral plasmid vector include, but are not limited to, polynucleotides encoding tumor necrosis factor (TNF) genes, such as TNF-.alpha.; genes encoding interferons such as Interferon-.alpha., Interferon-.beta., and Interferon-.gamma.; genes encoding interleukins such as IL-1, IL-1.beta., and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding epidermal growth factor (EGF), and keratinocyte growth factor (KGF); genes encoding soluble CD4; Factor VIII; Factor IX; cytochrome b; glucocerebrosidase; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (.alpha.1AT) gene; the insulin gene; the hypoxanthine phosphoribosyl transferase gene; the CFTR gene; negative selective markers or "suicide" genes, such as viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus; antisense c-myb oligonucleotides; and antioxidants such as, but not limited to, manganese superoxide dismutase (Mn-SOD), catalase, copper-zinc-superoxide dismutase (CuZn-SOD), extracellular superoxide dismutase (EC-SOD), and glutathione reductase; tissue plasminogen activator (tPA); urinary plasminogen activator (urokinase); hirudin; the phenylalanine hydroxylase gene; nitric oxide synthesase; vasoactive peptides; angiogenic peptides; the dopamine gene; the dystrophin gene; the .beta.-globin gene; the .alpha.-globin gene; the HbA gene; protooncogenes such as the ras, src, and bcl genes; tumor-suppressor genes such as p53 and Rb; the LDL receptor; the heregulin-.alpha. protein gene, for treating breast, ovarian, gastric and endometrial cancers; monoclonal antibodies specific to epitopes contained within the .beta.-chain of a T-cell antigen receptor; the multidrug resistance (MDR) gene; polynucleotides encoding ribozymes; antisense polynucleotides; genes encoding secretory peptides which act as competitive inhibitors of angiotensin converting enzyme, of vascular smooth muscle calcium channels, or of adrenergic receptors, and polynucleotides encoding enzymes which break down amyloid plaques within the central nervous system. It is to be understood, however, that the scope of the present invention is not to be limited to any particular therapeutic agent.

Detailed Description Paragraph Right (19):
In another embodiment, the retroviral vector is produced by introducing a wild-type retrovirus into a cell line which is resistant to lysis by human serum, and recovering the resistant retroviral vector from the cell line. In one embodiment, the retrovirus is selected from the group consisting of Rous Sarcoma Virus, RD114, BaEV, SSAV, FeLV-B, GALV, avian leukosis virus, and murine leukemia viruses, such

'as', for example, Moloney Murine Leukemia Virus or amphotropic murine leukemia viruses (MLV-A), including, but not limited to, strains 4070A and 1504.

Detailed Description Paragraph Right (22):
Applicants have found that when retroviral vectors are produced by introducing the above-mentioned retroviruses into the above-mentioned cell lines, the resulting retroviral vectors produced by the above-mentioned retrovirus/cell line combinations are resistant to inactivation by human complement proteins which are found in human serum.

Detailed Description Paragraph Right (23):
Alternatively, a retroviral vector may be produced by introducing into a pre-packaging cell (i.e., a cell including polynucleotides encoding gag and pol proteins), which is resistant to lysis by human serum, a plasmid vector including a polynucleotide which encodes an envelope protein, such as a retroviral env protein, and a retroviral plasmid vector including a 5' LTR and a 3' LTR, a packaging signal, and at least one polynucleotide encoding a protein or polypeptide of interest. The gag and pol retroviral proteins may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, gibbon ape leukemia virus (GALV), simian sarcoma associated virus SSAV, FeLV-B, human immunodeficiency virus, NZB virus, and avian leukosis virus. Alternatively, the gag/pol proteins may be modified or chimeric gag/pol constructs. The envelope may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, amphotropic murine leukemia viruses (MLV-A), Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, gibbon ape leukemia virus (GALV), SSAV, FeLV-B, human immunodeficiency virus, NZB virus, and avian leukosis virus. The envelope also may be an envelope which includes amphotropic and ecotropic portions. Alternatively, the env proteins may be modified or chimeric env constructs or obtained from non-retroviruses, such as vesicular stomatitis virus and HVJ virus.

Detailed Description Paragraph Right (24):
Thus, in accordance with another aspect of the present invention, there is provided a retroviral vector resistant to inactivation by human serum which has been produced by introducing into a pre-packaging cell line, which is resistant to lysis by human serum, a plasmid vector including a polynucleotide encoding an envelope protein which may be obtained from a retrovirus including those selected from the group consisting of Moloney Murine Leukemia Virus, Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, SSAV, FeLV-B, gibbon ape leukemia virus (GALV), amphotropic murine leukemia viruses (MLV-A), human immunodeficiency virus, NZB virus, avian leukosis virus; or an envelope which includes amphotropic and ecotropic portions; or may be obtained from a non-retrovirus, such as vesicular stomatitis virus or HVJ virus; or which may be a modified or chimeric env construct; and a retroviral plasmid vector including a 5' LTR, a 3' LTR, a packaging signal, and at least one polynucleotide encoding a protein or polypeptide of interest. In accordance with yet another aspect of the present invention, there is provided a packaging cell line for generating retroviral vectors resistant to inactivation by human serum which includes a polynucleotide encoding an envelope protein as hereinabove described. Preferably, the packaging cell line includes a first plasmid vector including a polynucleotide encoding the gag and pol retroviral proteins, and a second plasmid vector encoding an envelope protein as hereinabove described. The packaging cell line is resistant to lysis by human serum.

Detailed Description Paragraph Right (26):
As stated hereinabove, the polynucleotide encoding the gag and pol retroviral proteins may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, amphotropic murine leukemia viruses (MLV-A), Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, gibbon ape leukemia virus (GALV), SSAV, FeLV-B, NZB virus, and avian leukosis virus (ALV). Alternatively, the gag/pol proteins may be modified or chimeric gag/pol constructs. In general, the polynucleotide encoding the gag and pol retroviral proteins is contained in an appropriate plasmid vector. In one embodiment, the gag and pol retroviral proteins are obtained from Moloney Murine Leukemia Virus, and are contained in a plasmid known as pCRIPenv-, as described in Danos, et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 6460-6464 (1988).

Detailed Description Paragraph Right (27):
Another plasmid vector includes a polynucleotide encoding an envelope protein which may be obtained from any retrovirus, including, but not limited to, Moloney Murine

Leukemia Virus, Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, SSAV, FeLV-B, gibbon ape leukemia virus (GALV), amphotropic murine leukemia viruses (MLV-A), human immunodeficiency virus, NZB virus, and avian leukosis virus, or non-retroviruses, such as vesicular stomatitis virus and HVJ virus, or modified or chimeric env constructs. In one alternative, the envelope protein includes amphotropic and ecotropic portions. The plasmid vectors encoding the gag and pol proteins, and the env protein and the retroviral plasmid vector hereinabove described, then are transfected into a cell which is resistant to lysis by human serum to provide a helper-free packaging cell line which will generate retroviral particles resistant to inactivation by human serum and which include gag and pol proteins such as, for example, those hereinabove described, an envelope protein which may be obtained from any retrovirus, including, but not limited to, Moloney Murine Leukemia Virus, Rous Sarcoma Virus, the feline endogenous virus RD114, BaEV, SSAV, FeLV-B, gibbon ape leukemia virus (GALV), amphotropic murine leukemia viruses (MLV-A), human immunodeficiency virus, NZB virus, avian leukosis virus, or non-retroviruses, such vesicular stomatitis virus and HVJ virus, or a modified or chimeric env construct, or an envelope including amphotropic and ecotropic portions. Such cell line may further include a retroviral plasmid vector including at least one polynucleotide encoding a protein or polypeptide of interest, such as a therapeutic agent, which may include those hereinabove described. Thus, the packaging cell line becomes a producer cell line which generates a retroviral vector which is resistant to inactivation by human serum which also includes at least one polynucleotide encoding a therapeutic agent. Such retroviral vector particles may be employed in gene therapy procedures such as those herein described, and may be administered to a human host in dosages such as those herein described.

Detailed Description Paragraph Right (28):
In a preferred embodiment, the retroviral vector is produced in a cell line which is a human 293 cell line or a cell line derived from a human 293 cell line.

Detailed Description Paragraph Right (29):
Applicants have discovered surprisingly that, when retroviral vectors are produced from a human 293 cell line or a cell line derived from a human 293 cell line, that such retroviral vectors are resistant to complement inactivation by human serum, and that such complement resistance is not dependent upon the envelope employed, even when the envelope is an amphotropic envelope.

Detailed Description Paragraph Right (33):
The retroviral vectors, which are administered in vivo to an animal, can be produced by providing the human 293 cell line with the retroviral packaging function. Such packaging function may be provided by a replication competent retrovirus, or may be provided by a transient system which includes one or more expression vehicles (e.g., plasmid vectors) including polynucleotides encoding the gag, pol, and env proteins. Such functions also may be provided by stably transfecting the 293 cell line with one or more expression vehicles (e.g., plasmid vectors) including polynucleotides encoding the gag, pol, and env proteins. In addition to providing the 293 cells with the polynucleotides encoding the gag, pol, and env proteins, the 293 cells also are transfected with a retroviral plasmid vector as described hereinabove.

Detailed Description Paragraph Right (34):
The retroviral plasmid vector is transfected into the packaging cell line which is a human 293 cell line or a cell line which is derived from a human 293 cell line, whereby such packaging cell line becomes a producer cell line that generates retroviral vectors which are resistant to inactivation by human serum. The packaging cell line derived from human 293 cells may be generated, for example, by immortalizing the cell line by transformation with transforming proteins such as the simian virus 40 (SV40) large tumor antigen; alternatively, one may use immortalized 293 cell lines such as 293T or 293E. Once the appropriate 293 cell line is chosen, it is necessary to co-transfect these cells with a plasmid encoding retroviral gag/pol proteins such as, for example, Moloney Murine Leukemia Virus gag/pol proteins, along with a plasmid containing a selectable marker such as hygromycin. Individual hygromycin resistance clones are isolated and screened both for the presence of the gag proteins (p15, p12, p30, and p10) and for reverse transcriptase (RT) activity. Routinely the clone expressing the highest levels of p30 and RT is designated the pre-packaging cell line. To generate a packaging cell line, it is necessary to co-transfect the pre-packaging cell line with an env-containing plasmid and a plasmid containing a selectable marker different than the one used in the first step (e.g., puromycin resistance, etc.). If the env component is toxic to the cell, such as is the case with many fusogenic envelopes, then it may be necessary to

'express this from an inducible promoter. Clones then are screened, and an appropriate packaging cell line is chosen. Such packaging cell lines also may be prepared as disclosed in Pear, et al., September 1993, or in PCT Application No. WO94/19478, published Sep. 1, 1994. In one embodiment, the packaging cell line is the CAK8 (or Bing) cell line (ATCC No. CCRL 11554), which is an amphotropic envelope-expressing packaging line, whereby the resultant retrovirus generated by such cell line has an amphotropic envelope. In another embodiment, the cell line is the Bosc 23 (ATCC No. CCRL 11270), which is an ecotropic envelope-expressing packaging line, whereby the resultant retrovirus generated by such cell line has an ecotropic envelope.

Detailed Description Paragraph Right (37):
Thus, in accordance with another aspect of the present invention, there is provided a complement-resistant retroviral vector, which may be produced from a cell line selected from the group consisting of the HOS, TE671, HT1080, Mv-1-Lu, and human 293 cell lines, or a cell line derived from the HOS, TE671, HT1080, Mv-1-Lu, and human 293 cell lines, which includes an envelope selected from the group consisting of VSV-G envelope protein; Moloney Murine Leukemia Virus envelope; Rous Sarcoma Virus envelope; feline endogenous virus RD114 envelope; gibbon ape leukemia virus envelope; baboon endogenous virus envelope; simian sarcoma associated virus envelope; amphotropic murine leukemia virus (MLV-A) envelope; human immunodeficiency virus envelope; avian leukosis virus envelope; NZB viral envelopes; and HVJ virus envelope.

Detailed Description Paragraph Right (39):
The retroviral vectors are administered to an animal in vivo in an amount effective to produce a therapeutic effect in the animal. The animal may be a mammal, including human and non-human primates. The retroviral vectors may be administered systemically, for example, intravenously or intraarterially or intraperitoneally. The vectors also may be administered subcutaneously or intramuscularly. The retroviral vectors, which are resistant to inactivation by human serum, transduce cells in vivo, whereby the transduced cells express the therapeutic agent in vivo.

Detailed Description Paragraph Right (40):
The retroviral vectors are administered to an animal in an amount effective to produce a therapeutic effect in the animal. In general, the retroviral vectors are administered in an amount of at least 10.sup.5 cfu, and in general such amount does not exceed 10.sup.12 cfu. Preferably, the retroviral vectors are administered in an amount of from about 10.sup.6 cfu to about 10.sup.10 cfu. The exact dosage to be administered is dependent upon various factors, including the age, height, weight, and sex of the patient, the disorder being treated, and the severity thereof.

Detailed Description Paragraph Right (41):
The retroviral vectors are administered to the patient in a pharmaceutically acceptable carrier, such as, for example, a physiological saline solution. Other pharmaceutical carriers include, but are not limited to, mineral oil, alum, and lipid vesicles such as liposomes. The selection of a suitable pharmaceutical carrier is deemed to be within the scope of those skilled in the art from the teachings contained herein.

Detailed Description Paragraph Right (43):
The gene carried by the blood cells can be any gene which directly enhances the therapeutic effects of the blood cells. The gene carried by the blood cells can be any gene which allows the blood cells to exert a therapeutic effect that it would not ordinarily have, such as a gene encoding a clotting factor (e.g., Factor VIII or Factor IX) useful in the treatment of hemophilia. The gene can encode one or more products having therapeutic effects. Examples of suitable genes include those that encode cytokines such as TNF, interleukins (interleukins 1-12), interferons (.alpha., .beta., .gamma.-interferons), T-cell receptor proteins and Fc receptors for binding to antibodies.

Detailed Description Paragraph Right (44):
The retroviral vectors are useful in the treatment of a variety of diseases including but not limited to adenosine deaminase deficiency, sickle cell anemia, thalassemia, hemophilia, diabetes, .alpha.-antitrypsin deficiency, brain disorders such as Alzheimer's disease, and other illnesses such as growth disorders and heart diseases, for example, those caused by alterations in the way cholesterol is metabolized and defects of the immune system.

Detailed Description Paragraph Right (45):
In one embodiment, the retroviral vectors may include a negative selectable marker, such as, for example, a viral thymidine kinase gene, and more particularly, the Herpes Simplex Virus thymidine kinase (TK) gene. Such retroviral vectors may be administered to tumor cells (in particular to cancer cells) in a human patient in vivo. The retroviral vectors then transduce the tumor cells. After the retroviral vectors have transduced the tumor cells, the patient is given an interaction agent, such as gancyclovir or acyclovir, which interacts with the protein expressed by the negative selectable marker in order to kill all replicating cells (i.e., the tumor cells) which were transduced with the retroviral vector including the negative selectable marker.

Detailed Description Paragraph Right (46):
The retroviral vectors mentioned hereinabove also may be administered in an animal model for determining the effectiveness of a gene therapy treatment. For example, a retroviral vector, produced in a cell line which is resistant to lysis by human serum, and including a polynucleotide encoding a therapeutic agent, may be administered to animals of the same species in varying amounts. From determining the effectiveness of the gene therapy treatment in the animal, one may determine an effective amount of the retroviral vector to be administered to a human patient.

Detailed Description Paragraph Right (47):
In another embodiment, the cells which are resistant to lysis by human serum, which have been transfected with a retroviral plasmid vector such as hereinabove described, which includes one or more polynucleotides encoding a therapeutic agent, whereby such cells have become producer cells, are administered to a patient in vivo, whereby the producer cells generate in vivo retroviral vector particles including a polynucleotide encoding a therapeutic agent.

Detailed Description Paragraph Right (48):
Such an embodiment is applicable particularly to the treatment of tumors (including malignant and non-malignant tumors) such as, for example, brain tumors and head and neck tumors. For example, the producer cells may include a retroviral plasmid vector including a negative selectable marker. The producer cells then are administered to the tumor, whereby the producer cells generate retroviral vector particles including the polynucleotide encoding the negative selectable marker. The retroviral vector particles generated by the producer cells transduce the tumor cells, whereby the tumor cells produce the negative selectable marker. Upon administration of an interaction agent to the patient, the transduced tumor cells are killed.

Detailed Description Paragraph Right (49):
Alternatively, the retroviral vector may transduce eukaryotic cells, in vitro, whereby the eukaryotic cells are cultured in vitro for the in vitro production of the therapeutic agent, or, alternatively, the transduced eukaryotic cells may be administered to a host as part of a gene therapy procedure, whereby the transduced eukaryotic cells express the therapeutic agent in vivo in a host.

Detailed Description Paragraph Right (50):
In accordance with another aspect of the present invention, there is provided a method of identifying retroviral vectors that are resistant to inactivation by human serum comprising introducing a retrovirus into a cell line in which a resultant retroviral vector is to be produced. The resistance of the resultant retroviral vector to inactivation by human serum then is determined. The determination of the resistance of the resultant retroviral vector to inactivation by human serum may be made by methods described hereinbelow in the examples.

Detailed Description Paragraph Right (51):
In accordance with another aspect of the present invention, there is provided a method of producing retroviral vectors resistant to inactivation by human serum. The method comprises determining resistance of cells to lysis by human serum. Resistance of cells to lysis by human serum may be determined by methods such as those described hereinbelow. Retroviral vectors then are produced from those cells found to be resistant to lysis by human serum.

Detailed Description Paragraph Right (54):
The MFGnlslacZ genome (Ferry, et al., Proc. Nat. Acad. Sci., Vol. 88, pgs. 8377-8381 (1991) was introduced into NIH 3T3 and Mv-1-Lu cells by infection with lac Z (contained in Murine Leukemia Virus-A) produced from the .psi.CRIP packaging line containing MFGnlslacZ genome, as described in Tailor, et al., J. Virol., Vol. 67,

pgs. 6737-6741 (1993). MFGnlslacZ is a Moloney Murine Leukemia Virus based retroviral vector including a 5' LTR, a 3' LTR, a packaging signal, and a lacZ gene. After cell cloning by limiting dilution, clones which gave high titers of lac Z pseudotype in a pilot rescue experiment were selected. Lac Z pseudotypes containing helper virus were produced by infection of these cell clones with replication competent MLV-A 1504 strain. (Rasheed, et al., J.Virol., Vol. 19, pgs. 13-18 (1976); Sommerfelt, et al., Virology, Vol. 176, pgs. 58-69 (1990); Tailor, et al., 1993). Viruses were harvested in serum-free Opti-MEM (Gibco U.K.). On the day before harvest, cells were washed once with Opti-MEM and incubated in Opti-MEM at 37.degree. C. for 1 hour. The medium then was replaced with fresh Opti-MEM and cells were incubated overnight. Culture supernatant was harvested, filtered through a 0.45 .mu.m filter, aliquoted, and stored as virus stock at -70.degree. C. until use. All virus stocks had original lac Z titers ranging from 2.times.10.sup.4 to 4.times.10.sup.6 on appropriate assay cells.

Detailed Description Paragraph Right (83):
Plasmid pG1Na was derived from plasmid pG1. Plasmid pG1 was constructed from pLNSX (Palmer, et al., Blood, Vol. 73, pgs. 438-445). The construction strategy for plasmid pG1 is shown in FIG. 5. The 1.6 kb EcoRI fragment, containing the 5' Moloney Murine Sarcoma Virus (MoMuSV) LTR, and the 3.0 kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 (FIG. 7) by the insertion of the 1.6 kB EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 (FIG. 7) consists of a retroviral vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender, et al. 1987), a 54 base pair multiple cloning site (MCS) containing, from 5' to 3', the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindIII, ApaI, and ClaI and a 3' portion of MoMuLV from base pairs 7764 to 7813 (numbered as described (Van Beveren, et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985) (FIG. 6). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo.sup.r gene, the .beta.-galactosidase gene, the hygromycin.sup.r gene, and the SV40 promoter.

Detailed Description Paragraph Right (90):
This example compares the sensitivity of retroviral vectors including pG1Na containing either an amphotropic envelope produced from PA317 cells, an amphotropic envelope from human CAK8 cells, or a VSV-G envelope from a stable mouse cell line (gp7C).

Detailed Description Paragraph Right (91):
The CAK8 cell line (ATCC No. CCRL 11554) was derived from the 293T cell line (Pear, et al., Proc. Nat. Acad. Sci., Vol. 90, pgs. 8392-8396 (September 1993). The CAK8 cell line includes a polynucleotide encoding a retroviral envelope derived from the amphotropic 4070A retrovirus, wherein the polynucleotide sequence encoding the 35 C-terminal amino acids of 4070A retrovirus is replaced with a polynucleotide encoding the 35 C-terminal amino acids of an ecotropic virus. A clone, termed 293T/17, was isolated from a 293T population (Du Bridge, et al., Mol. Cell. Biol., Vol. 7, pgs. 379-387 (1987)) that produced retroviral supernatants capable of infecting NIH 3T3 cells at titers greater than 10.sup.6 /ml following transient transfection with wild-type Moloney virus (pZap) (Shoemaker, et al., J. Virol., Vol. 40, pgs. 164-172 (1981)), and a .beta.-galactosidase-expressing retroviral vector pBND8 (Pear, et al., 1993). The gag-pol expressing plasmid, pCripEnv- (Danos, et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 6460-6464 (1988)), which contains a mutation in the envelope region, lacks the packaging site, and replaces the 3' LTR with the SV40 poly (A) site, was transfected into 293T/17 cells along with a plasmid conferring hygromycin resistance. (Bernard, et al., Exp. Cell. Res., Vol. 158, pgs. 237-243 (1985)). Individual clones were selected and tested for reverse transcriptase activity (Goff, et al., J. Virol., Vol. 38, pgs. 239-248 (1981)), and one clone, Anjou 65, had the highest reverse transcriptase activity. The amphotropic envelope expressing construct, pCripAMgag-(Danos, et al., 1988), which contains mutations in the gag region, lacks the packaging site, and replaces the 3' LTR, was transfected into Anjou 65 cells along with a plasmid expressing the gpt resistance gene. (Jasin, et al., Genes and Dev., Vol. 2, pgs. 1353-1363 (1988)). Individual clones were isolated and tested for the ability to produce high titer .beta.-galactosidase-expressing retroviruses. One clone produced .beta.-gal retrovirus with a titer in excess of 10.sup.6 /ml following transfection with pBND8.

Two rounds of limiting dilution subcloning were performed subsequently, giving rise to the CAK8, or Bing cell line.

Detailed Description Paragraph Right (92):
Amphotropic G1Na retroviral supernatant was generated by transient transfection of the CAK8 packaging cell line or of PA317 cells as described in Pear, et al., 1993. Retroviral supernatant was harvested 48 hours post-transfection. VSV-G pseudotyped G1Na retroviral vector (provided by Dr. Elio Vanin, St. Jude Children's Research Hospital, Memphis, Tenn.) was prepared from a stable mouse cell line (gp7C) containing a VSV-G envelope under a tetracycline inducible promoter and an ecotropic gag/pol.

Detailed Description Paragraph Right (94):
A retroviral vector genome containing the neo.sup.R gene (G1Na) was introduced into GP7C cells by exposure to culture medium from a producer clone (G1Na.40, Genetic Therapy, Inc., Gaithersburg, Md.) generating amphotropic vector particles. (G1Na.40 was generated by transducing the PA317 cell line with pG1Na). Individual clones were isolated by G418 selection and culture media from each was assayed for content of vector RNA by RNA slot blot analysis. A clone designated GP7CN having the highest apparent titer, was selected.

Detailed Description Paragraph Right (103):
As shown in FIG. 10, when the VSV-G pseudotyped G1Na retroviral vector was incubated with human serum for 30 minutes, a 35-fold decrease in titer was observed. No titer loss was seen with heat-inactivated fetal bovine serum, and a minimal loss of titer (approximately 2-fold) was observed with heat-inactivated human serum.

Detailed Description Paragraph Right (104):
In contrast to the results obtained with the VSV-G pseudotyped retroviral vector, amphotropic G1Na vector produced from the CAK8 cell line was completely stable in the presence of human serum (FIG. 11), whereas the G1Na vector produced from PA317 cells resulted in a 50-fold loss in titer after incubation with human serum for 30 minutes (FIG. 12).

CLAIMS:

1. A retroviral vector resistant to inactivation by human serum, said retroviral vector having been produced by a producer cell resistant to lysis by human serum, wherein said producer cell includes:

(i) a polynucleotide encoding a retroviral envelope protein, said polynucleotide encoding said envelope protein being obtained from a virus selected from the group consisting of feline endogenous virus RD114, BaEV, SSAV, FeLV-B, NZB virus, avian leukosis virus, and HVJ virus, and

(ii) a retroviral vector including a 5' LTR, a 3' LTR, a packaging signal, and at least one polynucleotide encoding a protein or polypeptide of interest, wherein said producer cell does not include the entire viral RNA of feline endogenous virus RD114, BaEV, SSAV, FeLV-B, NZB virus, avian leukosis virus, or HVS virus, and wherein said producer cell is derived from a cell selected from the group consisting of the TE671, HT1080, MV-1-Lu, and human 293 cell lines, and cell lines derived from the TE671, HT1080, Mv-1-Lu, and human 293 lines.

2. The retroviral vector of claim 1, wherein said envelope protein is obtained from feline endogenous virus RD114.

3. The retroviral vector of claim 1, wherein said envelope protein is obtained from BaEV.

4. The retroviral vector of claim 1, wherein said envelope protein is obtained from SSAV.

5. The retroviral vector of claim 1, wherein said envelope protein is obtained from FeLV-B.

6. The retroviral vector of claim 1, wherein said envelope protein is obtained from NZB virus.

7. The retroviral vector of claim 1, wherein said envelope protein is obtained from

avian leukosis virus.

8. The retroviral vector of claim 1, wherein said envelope protein is obtained from HVJ virus.